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Haematopoietic stem cells—present perspective and future directions

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Abstract

Haematopoietic stem cells have been and will continue to be a source of interest to clinicians as well as cell biologists Haematopoietic stem cells are the most well characterized adult stem cells in humans There are different sources of Haematopoietic stem cells like bone marrow, peripheral blood and umbilical cord blood, that are being clinically used for bone marrow transplantation Other sources like embryonic stem cells and fetal liver stem cells are limited to the experimental settings

Advances in technology are fuelling interest in developing safer and better haemopoietic stem cell transplantation procedures for hitherto incurable diseases Various stem cell banks and registries are coming in vogue for easy availability of these stem cells

Simultaneous development and interaction of various technologies will allow invitro expansion of stem cells, which may enable better engraftment and faster post transplantation recovery

Genetic manipulation of these stem cells for corrective gene therapy is also another area of great future potential.

Many people have demonstrated transdifferentiation of these hematopoietic stem cells to various other tissue types like cardiomyocytes, neuronal cells, etc. The use of these transdifferentiated hematopoietic stem cells may allow repair of damaged and degenerated cells.

(**Key words**: haematopoietic stem cells/transplantation/transdifferentiation/exvivo expansion.)

Introduction

A hematopoietic stem cell (HSC) is defined as a cell with extensive self renewal and proliferative potential, coupled with the capacity to differentiate into the progenitors of all the blood lineages, that is, erythrocytes, neutrophils, eosinophil, and basophil granulocytes, mast cells, monocytes and macrophages, platelets, B lymphocytes, T lymphocytes, natural killer cells, and dendritic cells. The concept of a HSC is not new to the hematologist For 50 years there has been a lot of debate as to the nature, number and restrictiveness in the differentiation capability of these stem cells. The monophyletic theory predicted the presence of pluripotent stem cells from which all differentiated blood cells arise, on the other hand the polyphyletic theory surmised the presence of multiple stem cells with restricted differentiation capability. Though initially there was a lot of disagreement between monophyletists and polyphyletists, advances in modem cellular biology proved that neither was wrong. There actually exists a hierarchy within the stem cell compartment, beginning with pluripotent stem cells[1] to multipotent stem cells[2]. This hierarchy was established using development of monoclonal antibodies against two categories of antigens which develop on the haematopoietic cells (i) Antigens that are maturity specific (i.e. TdT. HLA DR etc) (ii) Antigens which are lineage specific (eg. CD 33, CD13 for Myeloid, CD45 for leukocytes, CD71 for erythroid lineage, CD42 for megakaryocytic etc).

Antigens expressed on immature precursors of haemopoietic cells drew more attention for the detection of HSCs; CD34 was found to be one such antigen and is now extensively used for enumerating, isolating and studying the properties of HSCs[3]. Development of flow cytometry with cell sorting techniques and development of more simple technologies to isolate haemopoietic stem cells[4] were additional advancements in this field. Before we discuss any further we have to look into the fact why HSCs drew so much attention? From the beginning of the 20th century it became apparent that there are many diseases of the haemopoietic system where stem cells are defective or deficient eg hypoplastic anaemia, myelodysplastic syndrome, pure red cell aplasia etc. With the advent of the X ray machine, radioisotopes etc it also became

clear that radiation could damage the marrow and cause a picture like aplastic anaemia In the early 50s, laboratory experiments demonstrated that splenic shielding or intravenous administration of marrow cells protected animals from lethal irradiation From 1960 onwards intensive research in this area has lead to successful allogenic bone marrow transplantations[5] Development of recombinant, lineage specific stem cell growth factors in pharmacological amounts and more potent immunosuppressive drugs including cyclosporin, FK - 506, Mycophenolate Mofetil, Deoxyspergualin, Antilymphocytic globulin, Rapamycin improved the outcome of allogeneic bone marrow transplantation so that it became a clinical reality, helping thousands of patients to live

However, there were many more areas of biology, that needed to be developed before successful allogenic hematopoietic stem cell transplantation (HSCT) could be done One of these was a better understanding of the immunobiology of stem cell transplantation and tissue typing techniques These are interesting areas in their own right Since the 1990's with the availability of growth factors (e g G-CSF) and improvement in the technology of cell apheresis it has been possible to harvest an adequate number of transplantable HSCs from a donor's peripheral blood[6] without subject to an anaesthetic procedure and bone marrow harvest This has lead to an exponential increase in HSCT and to date almost 2,00,000 allogeneic HSCT have been done worldwide

Sources of HSCs:

The transplantation of marrow to rescue patients from lethal radiation or chemotherapy or to replace abnormal marrow has evolved over past three decades from an act of desperation administered to patients with end stage disease to an acceptable and in some instances, first-line therapy administered early in the course of a variety of malignant and nonmalignant disorders Advances in the transplantation biology and supportive care have made the evolution possible and have helped to usher in the modern era of marrow transplantation

A variety of sources are being utilized for the collection of HSCs for transplantation procedure These include bone marrow, peripheral blood especially following mobilization and umbilical cord blood.

Bone marrow :

Bone marrow has served as the traditional source of HSCs for transplantation The procedure for harvesting the bone marrow from human donors was first described in

1970[7] and has not changed significantly since Marrow is aspirated from posterior iliac crests under either general or regional anesthesia in a sterile environment The cell dose required for stable engraftment has not been defined with certainty but the present recommended dose is as follows for an HLA matched sibling and unrelated graft, a minimum total of $3x10^8$ nuclear cells per kilogram of the recipient's body weight, if allogenic marrow is to be T-Cell depleted or if an ABO mismatch exists between donor and the recipient, a minimum of $4x10^8$ cells/kg is required, if tumor cell purging (using monoclonal antibody + complement, CD34+ cell selection or chemotherapeutic agents) is Performed on an autologous harvest, $6x10^8$ cells/kg may be needed[8,9]

Complications of marrow harvesting Bone harvesting is a safe procedure with minimal risk of complications A review of over 3000 records of histocompatible bone marrow donors by the International Bone Marrow Transplant Registry and the Marrow Transplant team at Fred Hutchinson Cancer Research Center determined that overall incidence of life threatening complications associated with the harvest procedure was 0 27% and most of them attributable to the risk of general anesthesia[10,11] However all donors had pain at the site of harvest for several days postoperatively, though less than 0 5% of the donors experienced pain persisting for more than a week or more

Peripheral blood:

HSCs circulate in the peripheral blood of normal individuals at extremely low levels However it is possible to increase the number of peripheral blood stem cells (PBSC) by using hematopoietic growth factors and or chemotherapeutic agents - termed as 'mobilization'

A number of Hematopoietic growth factors have been found to be effective mobilizing agents, including G-CSF, GM-CSF, IL-3 and thrombopoietin (TPO) [12,13] The most common mobilizing regimen includes administration of G-CSF at 10ug/kg/day, followed by apheresis on the fourth and fifth days[14] A variety of other regimens are available using CSFs alone or in combination with chemotherapy Other growth factors have also been used in combination with G-CSF Stem cell Factor (SCF) or C-kit ligand has been found to synergize with G-CSF in mobilizing CD34+ cells[15] Another cytokine Flt-3 ligand has been shown to increase the mobilization of HSCs in a time dependent manner with G-CSF[16]

HSC mobilization was first described as a procedure following myelosuppressive therapy[17] A variety of myelosuppressie chemotherapy regimens can mobilize HSCs

into peripheral blood Commonly used regimens include cyclophosphamide alone, in single dose of 4-7g/m2 or other agents like Adriyamycin, carboplatin, Taxol, etoposide, ifosfamide, daunorubicin, cytosine arabinoside, 6- thiogaunine, either alone or in combination have been employed[18-20] The regimens induce profound myelosuppression in patients, with white cell counts dropping below 100 cells/mm^3 7-14 days after chemotherapy This is typically followed on days 10-21 by rebound increase of the circulating leukocytes above baseline levels, which is also associated with an increase of HSCs in the peripheral blood Ideally, the patient's malignancy is sensitive to the chemotherapy, which also provides antitumour effect there by reducing the risk of mobilization of tumor cells Disadvantages of employing chemotherapy alone for mobilization include the necessity to submit the patient to the potential side effect of myelosuppression, uncertainty regarding optimal timing for collection, wide variability in the extent and duration of myelosuppression, and an inability to predict the success of mobilization in a given patient

Socinki et al first demonstrated that administration of GM-CSF after chemotherapy increased circulating CFU-GM[12] Many investigators have since then reported that mobilization employing a combination of chemotherapy and followed by growth factor (GM-CSF or G- CSF) administration is more effective than either chemotherapy or growth factor alone[19,21-23] The combination typically results in a 50-75 fold increase in circulating CFU-GM, and 10-50 fold increase in CD34+ cells[21,23]

Though the optimal methodology for mobilization of PBSC has not been defined yet, the absolute number of CD34+ cells/ kg recipient weight has proven to be a reliable and practical method for determining the adequacy of the stem cell product The optimal dose is controversial However most of the transplant centers have observed that stem cell content of more than $2x10^6$ CD34+ cells/ kg result in rapid hematopoietic recovery[24] The minimum dose required for hematopoietic engraftment has not been defined, but doses below $1x10^6$ CD34+ cells/kg are inadequate The speed of engraftment and the overall outcome also depends on the prior chemotherapy and or radiotherapy received Patients exposed to prior chemotherapy for more than 24 months require higher doses of CD34+cells to ensure rapid engraftment (5 vs $2x10^6$/kg)[25]

The clinical significance of the tumor cells in the autologous PBSC graft has generated lot of debate There is no doubt that an autologous PGSC graft contain tumor cells especially when detected by a sensitive technique like PCR Hence methodologies to purge the PBSC graft of the possible tumor cell contamination have been designed These include ex-vivo treatment of the PBSC graft with the chemotherapeutic agents[26], monoclonal antibody and complement mediated methods

including positive selection of stem cells[27] or removal of tumor cells[28] These techniques have resulted in reduced tumor burden and subsequent relapse rate in small studies[29-20], but there have been no prospective clinical trials assessing the role of purging in autologous transplantation

Though role of PBSC in autologous setting is well established its role in allogenic settings is yet to be established As this procedure obviates the need for general anesthesia and its associated risks, it is being increasingly used in allogenic settings The concern with the use of PBSC in the allogenic setting is that large numbers of T - cells in the graft may increase the risk of graft- versus host disease However despite large number of T cells in the graft, initial studies have not shown an increase in the acute graft versus host disease[31,32], but the incidence of chronic graft versus host disease may be highe[33] The relative merits of marrow versus G-CSF mobilized PBSC in allogenic transplantation are currently being evaluated in randomized prospective clinical trials

Umbilical cord blood

1989 Gluckman *et al* first reported the successful use of umbilical cord blood (UCB) stem cells for transplantation in a child with Fanconi's anemia[34] Since then the potential of UCB as a source of HSC for transplantation rapidly became an area of intense clinical and scientific interest More than 1500 transplants have been performed till date using UCB as the source of HSCs

Over last one and half-decade considerable progress has been made in the understanding of the biology of the DCB HSCs It has been demonstrated that DCB contains a high proportion of primitive hematopoietic cells, including multipotent CFC s well as in vivo SCID repopulating cells, than in adult BM[35] Furthermore, in vitro studies have shown that DCB-derived HSCs possess higher proliferation and expansion potential than their BM counterparts[36] It is a good source of stem cells for gene therapy It also has shown a potential for transdifferentiation[38-40]

Clinical results of UCB transplantation have been encouraging It has some distinct advantages 1) can be easily and rapidly procured 2) has a significantly low risk of infections like cytomegalo virus and Epstein-Barr virus, compared BM 3) and no risk of donor attrition with some potential advantages like 1) reduced risk of GVHD 2) and the ability to expand donor pools in targeted ethical and recial minorities currently underrepresented in all marrow donor registries[41-43]

However there are some potential disadvantages with umbilical cord blood as well 1) longer time for neutrophil and platelet recovery predisposing patients to

increased risk of infection and bleeding post transplant 2) insufficient number of HSCs limiting it's widespread use in adults 3) a lower risk GVHD might translate into higher risk of relapse (due to reduced graft versus leukemia effect) [44,45]

Keeping all these points in mind cord blood collection and transplantation from a healthy sibling donor can be considered when an older child in the family requires transplantation UCB should be considered when allogenic transplantation is the treatment of choice for a child who does not have an HLA identical sibling donor or a well-matched unrelated adult volunteer donor

Evolving techniques of HSCT

Successful haemopoietic stem cell transplantation involves

(a) A myeloablative conditioning regimen
(b) Adequate haemopoietic stem cell infusion
(c) Taking adequate protection against bacterial, viral and fungal infection
(d) Prophylaxis against Acute Graft -Versus- Host Disease (GVHD)
(e) Treatment of complications as they arise

Each of these areas has undergone some changes over the last 3 years, so that starting with a morbidity of 40-50% in the first 3 months of transplantation many centers today report a 15-20% mortality for HLA matched sibling HSCT This improvement has come not through a quantum leap in any of the management areas but with better understanding of the engraftment process, together with anticipation and early management of the complications as a multidisciplinary approach

Conditioning regimens evolved from those containing total body irradiation (TBI) to fractionated TBI and hyper fractionated TBI[46,47], followed by hybrid regimen containing TBI and chemotherapy[48] to totally chemotherapy[49] The various types of regimen has allowed the clinician to select that is suitable regimen for a given patient depending on disease status, drug sensitivity, nature of the disease, type of transplantation etc, thus maximizing the patient's chance of achieving a cure A recent addition to this advancement is the development of non-myeloablative regimens[50,51] using much smaller dose of TBI with or without fludarabin, cyclophosphamide, and antilymphocyte globulin etc in various combinations This kind of regimen allows transplantation in elderly patients and as the marrow is not totally ablated recovery is

better although the risk of GVHD is higher This development also substantially reduces the cost of the transplantation process

Advancement in GVHD prophylaxis with cyclosporine, Mycophenolate Mofetile, FK-506, rapamycin etc has controlled acute GVHD to large extent so that Grade III and IV acute GVHD which used to affect a substantial number of patients is now seen infrequently[52] Advances in prophylaxis against various infections like Herpes simplex, CMV etc, routine use of IvIgG and surveillance for CMV virus activation has also reduced this morbidity

Enlarging Vistas of clinical application

In late 1960 s early 1970 s when haemopoietic stem cell transplantation came into being as a clinical reality, its application was restricted to severe aplastic anemia and relapsed acute leukemia patients Subsequently its application was expanded to include various hematological and non-hematological disorders like high risk acute leukemias, chronic myeloid leukemias, haemoglobinopathies including thalassemia and other immunodeficiency disorders like severe combined immune deficiency (SCID) Many inherited metabolic disorders like mucopolysaccharidosis, mucolipidosis, glycogen storage diseases can be treated by HSC transplantation and if done sufficiently early can halt the progression of these diseases [53,54]

Mean while continuous research in the area of autologous haematopoietic stem cell transplantation showed that it can be used to treat various hematological malignancies after purging the autologous stem cells of tumor cell by using chemical, physical or immunological techniques[26,27,28] High does chemotherapy and autologous hematopoietic stem cell transplantation have been employed with or without purging techniques to treat a number of solid tumors, with largest experience being in breast cancer Autologous hematopoietic stem cell transplantation in metastatic and high risk stage II and III disease have shown improved survival compared to historical controls[55,56] Similar studies in other solid tumors like advanced germ-cell tumor and neuroblastoma also have shown better disease free survival [57,58]

In patients with autoimmune disorders who received stem cell transplantation for the treatment of another disease, improvement in the disease activity was observed which is thought to be due to the effect of conditioning regimen on immunological system Early reports using Autologous HSC transplantation for the treatment of multiple sclerosis and other intractable autoimmune diseases have been encouraging[59,60] However other reports have shown early recurrence or no benefit[61] Hence further evaluation is required, especially concerning the source of graft

(allogenic versus Autologous), preparative regimen, and the requirement for and extent of T-cell depletion in the autologous setting

Ex-vivo expansion of HSCs

Over the last decade there have been several attempts to expand the stem cell number in vitro[62,63] using various Cytokine cocktails, Culture techniques and concept of modern Biotechnology

However till date a fool proof method of expanding stem cell number, keeping its initial biological properties intact have not been obtained This does not mean that it will never be possible to achieve our objective invitro[64] The very fact that the same operation goes on in vivo i e inside every human body suggests that we have not got the conditions right Either we are not using the appropriate cocktail of growth factors, in the correct amount and in right sequences or, more likely, we are not able to create the required cellular microenvironment in vitro The stem cell requires a proper supplying structure (i e Ground substance, type of collagen and other adhesion molecules) to make the stem cell proliferate without understanding their journey towards the path of differentiation Development of modern polymer sciences and molecular biology and cell culture technologies will eventually allow us to develop the haematopoietic Inductive microenvironment to support stem cell proliferation in an industrial scale in the same way as we produce various microchips today! And once we achieve this, the ability to supply HSCs as per requirement may be possible, though not necessarily at a cost, which is affordable to all

HSCs as a target for gene therapy

Since the first replication-defective retroviral vector was described in 1983, offering safe and effective route of transfer of exogenous genes to non-transformed human cells[65], hundreds of clinical trials of gene therapy have been undertaken. Laboratory and clinical hematologists have been critical to the development of gene transfer technologies, studying diseases, which are obvious and early targets for corrective gene therapy HSC are attractive targets for gene therapy as they are readily available from autologous sources, including peripheral blood and cord blood at birth, they can be relatively easily manipulated ex-vivo and they have a good proliferative potential Large number of disorders including leukemias and other cancers, infectious diseases, and a broad list of genetic diseases may be responsive to gene alteration of HSC and their progeny[66,67]. However very few gene therapy trials using HSCs have shown unequivocal benefits till date, one being on children with (SCID) treated with

autologous bone marrow modified to carry corrective gene[68] and other one being on patients with Adenosine Deaminase (ADA) - deficiency SCID[69] However two major adverse events appear to be direct result of the transferred gene reported have resulted in the temporary halt of all the trials[70] Hence though this field of gene therapy has promising future, we still have a long way to go in this direction

Concept of transdifferentiation a new paradigm in stem cell biology

It was a dogma in biology that once pluripotent stem cells have made the decision to develop down the line of haematopoeitic differentiation lineage then it is not possible to make these cells differentiate towards another differentiation pathway i e liver cells, neural cells etc However this dogma has now been challenged from some of the clinical and experimental evidences [72,73,74]

The ability of human progenitor cells derived from bone marrow to generate nonlymphohematopoietic tissue has been studied in allogenic sex-mismatched settings The initial studies were from Horwitz and colleagues[75] who claimed that stem cells derived from bone marrow led to improved osteogenesis in children with osteogenesis imperfecta Subsequently two groups independently reported the presence of donor cells that were positive for Y chromosome in liver tissue after male-into-female bone marrow transplantation[76,77] Indications that circulating stem cells can contribute to the formation of solid organ tissue derive from studies of solid-organ transplantation Quaini et al[78] and Muller et al[79] have reported male chimerism in heart allografts from female donor

This process of transdifferentiation has now attracted tremendous attention because of its potential application in diverse clinical situation like myocardial revascularisation, peripheral vascular diseases, generation of cardiomyocytes in the event of loss of heart muscles leading to severe congestive cardiac failure, generation of neural tissue in Parkinson's disease, Alzheimer's disease, pancreatic β cells in certain subsets of diabetics etc However though the observations that organ-specific cells originating from circulating blood colonize solid organ tissues suggest that tissue repair may be feasible, for it to become a potential clinical reality we require three conditions First, the concentration of the stem cells at the site of tissue regeneration must be sufficient, which may be achieved by either cytokine mobilization or by direct delivery of these cells to the site of injury Second, appropriate signals from the site of damaged tissue must direct exogenous stem cells to differentiate into tissue specific cells and third, these differentiated cells should survive for sufficient period and acquire the function of the particular organ

Marriage of cell technology and molecular biology

Continued improvement in HSCT not only requires a multispecialty approach but at basic levels it involves a marriage of cell technologies and techniques of molecular biology Already in the area of cell technology, it is possible to isolate different types of cells i e stem cells and others from marrow or peripheral blood using different immunological techniques[27] These techniques allow functional cells to be separated and kept as such Improvement in cryopreservation allows the stem cells to be frozen for indefinite periods For unrelated and related stem cell transplantation using molecular HLA typing to closely match the donor- recipient pairs Various molecular technologies to diagnose Chimerism, viral (CMV) infections are now routinely used The next level of advancement will require the use of molecular techniques to develop desired direction of immune modulation following stem cell transplantation[80] This may involve using a patterned pulse of cytokine cocktails or modify the dendritic cells from the donors using a synthetic peptide or segment of DNA, which is presumed to be carrying disease Molecular biological techniques and information from human genome project is increasingly being employed to develop gene products, which could be useful in stem cell modulation and post transplantation reconstitution of various stem cell compartments as fast as possible Quick immune reconstitution is a necessity for successful stem cell allografting Fusion of technologies may allow development of hybrid growth factor or cytokine molecules with several desirable attributes Development of P1 × 4321 which was hybrid molecule between GM - CSF and IL-3 is an earlier and relatively unsuccessful attempt in this respect

Demand and Supply of haemopoietic stem cells

As the safety of the haemopoietic stem cell transplantation improves with the improvement of technologies and graded levels of Chimerism in a balanced state becomes achievable with the capability of transdifferentiating a haemopoietic stem cell on the clinically desirable trans differentiation programme, a totally new era of haemopoietic stem cell transplantation will enter

In the wake of this revolution, several problems and challenges will also be tackled One of the major problems will be demand versus supply of these stem cells and entry of market economics in this area with possible erosion of ethical values[82] The ways by which the exponentially increasing demand could be achieved are

(a) Establishment of more stem cell registries allover the world in the same way as voluntary blood donors have been recruited

(b) Development of technologies to isolate adequate number of such cells from the donated blood units (At present this is not possible but successful stem cell transplantation was possible using a liter of donor blood)

(c) Using umbilical cord blood as a source of haemopoietic stem cells

(d) Using aborted fetuses as source of stem cells (Fetal Liver)

(e) Using super numerary embryos as a source of pluripotent and totipotent stem cells

These embryos are obtained from hospitals, clinics and laboratories involved in assisted reproductive techniques

(f) Techniques of Ex-vivo expansion of stem cells with maintenance of its initial properties

Stem cell banks - the future

It seems today that in near future the stem cell bank is the way forward An immediate application will be in the form of stem cell banks involving umbilical cord blood cells Several such banks[83,84] are already in existence but their number needs to increase enormously Umbilical cord blood stem cell banks will be the most cost effective banks in the present scenario HSC donor registry has the advantage that this registry remain in Silico hence cost wise it is relatively cheaper to maintain However adult donors may not always be traceable and even when they are traceable they may not like to donate stem cells when the need arises Hence in effect only 1 in 5 - 10 donors are realistically available for haemopoietic stem cell transplantation In our present level of technology when unrelated stem cell transplantation is contemplated, best results are obtained when total HLA match between the donor and the recipient is possible However for umbilical cord blood stem cells one or two HLA antigen mismatch gives similar results to that of total HLA match hence applicability of cord blood stem cells are likely to be more

Moreover as said previously cord blood stem cells are immediately available and availability to the recipient is not dependant on the whims and fancies of the donor If these stem cell banks store different varieties of stem cells and undertake Invitro expansion programme and later upgrade their capability to stem cell directed gene therapy programme then these stem cells banks will be the foundation for a totally different kind of future in biotherapeutics

Conclusion

Thus the concept of HSC has now come full circle From monophyletic-polyphyletic dualistic to modern hierarchy of the stem cell balancing all the contrasting views Advances in cryobiology, biotechnology, molecular biology, tissue engineering and *in vitro* cultivation techniques have allowed these stem cells to be collected, preserved, altered to suit our choice, expanded ex vivo and used for various clinical purpose of tissue engineering and repair Modern biology has also challenged the old paradigm that HSCs can only produce haemopoietic cells (i e differentiated haemopoietic cells like erythroid, myeloid, lymphoid and cells of megakaryocytic lineage) by demonstrating their capability of trans differentiation to other type of cells e g neural, hepatic, muscle, and endothelial cells in response to different environmental stimuli It is this trans differentiation capability of these cells that is fuelling further imagination in stem cell research and enlarging its vistas of clinical applicatic to various non hematological disorders like ischaemic heart disease, Parkinson's disease, peripheral vascular disease to name a few In future stem cells and cell lines obtained from them can be used to study drug toxicity and will also allow a transgene expression if properly manipulated In the not too distant future it may be possible to expand the stem cells in artificial environment and make them differentiate into desired lines like RBC, WBC etc Hence artificial development of stem cell culture and differentiation may allow us to develop red cells in vitro with rare blood groups for transfusion

These stem cells are also likely to be a suitable vehicle for innumerable ex vivo gene therapy protocols in future with promise to cure many hitherto intractable genetic disorders

Stem cell research may be suitably integrated into the emerging subject of genomics Genomics is the entire process of developing gene based therapies and drugs starting from sequencing to analysis and interpretation leading to drug development

HSC usage, particularly in the area of corrective gene therapy is an evolving field Though early success in some of the diseases have been recorded, development of a lymphoproliferative disorder in one such patient after corrective gene therapy in immunodeficiency have introduced an element of caution in this field Haematopoietic stem cells with its counterparts of other kinds of stem cells for therapeutic purposes are emerging as an area of regenerative medicine The progress in this area may be slow but it is inexorable. We have still may things to learn from the biology of stem cells Our tools of gene manipulation for HSCs are still very crude and needs

considerable refinement Graft versus host disease remains a formidable problem for allogenic HSC therapy A patient with severe graft versus host disease often may wonder whether his/her original disease was better! We need a better understanding and therapy to control this condition

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In vivo effects of mammalian insulin on the islet cells and metabolic levels in a fish, *Barbus conchonius* Ham.

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Abstract

A- and B- types of cells are uniformly distributed in the large 'principal' islet of *Barbus conchonius* Conspicuous degranulation and degeneration of B-cells were observed, followed by regressive changes in A- and B-cells and vacuolization of the islet, after treatment with insulin A significant hypoglycemia 8 hr post injection was observed Amino nitrogen, free fatty acids and organic PO_4 were notably depressed at time intervals varying 2 8 hr after insulin treatment There was significant, yet recoverable, reduction in blood cholesterol level The glycogen in liver and skeletal muscles registered an increase above control levels Most fish were able to recover from insulin shock and various parameters returned to normal levels usually within 48-72 hrs The response of islet cells is in correlation with the metabolic alterations in the tissues, and loss of secretory granules appears to be a secondary feature The results suggest that even heterologous insulin is remarkably effective in this species

(**Keywords** principal islet/ insulin/ hypoglycemia/degranulation/ hypoaminoacidemia/ hypocholesterolemia/ FFA/organic PO_4)

Introduction

The endocrine component of the pancreas in several teleosts consists of one or more macroscopically visible prominent nodules of tissue, called the 'principal' islets, that are more or less segregated from the exocrine part This has been called the 'islet organ' in the eel, *Anguilla rostrata,* due to its distinctive features[1] Usually, several smaller accessory islets are also present in the mesentery Role of islet cells in blood glucose regulation of fish and functional significance of the cellular components have been studied by some investigators[2-7] A perusal of the literature reveals that insulin affects serum glucose levels in teleostean fishes[8-12] and that it may promote protein synthesis[13-14] Bovine insulin causes a reduction in the plasma free fatty acids and phospholipids, but its effects upon tissue glycogen level are variable[9] In the present investigation, histo-pathological alterations in the islet cells and metabolic changes in

the tissues of *B conchonius* have been studied to see how far the heterologous insulin is effective in this cyprinid fish as compared to the air breathing teleosts studied earlier

Material and Methods

Adult *Barbus conchonius*, weighing 4 5-5 g, were obtained from Nainital lake and acclimatised to laboratory conditions for two weeks prior to use at 18±1°C water temperature and natural photoperiod Fish were divided into groups forming experimental and control animals Mammalian insulin (Boots Pure Drug Company (India) Ltd , Bombay) was injected into the epaxial musculature at two dose levels, 200 and 400 IU/kg body weight The control fish received 0 58 % saline in place of the hormone Details of experimental procedures have been described earlier[6,15] At autopsy, pancreatic islets were carefully removed from the experimental and control fish, fixed in Helley's fluid or mercuro-formol, and stored in 70% alcohol Sections were cut at 5-6 µm and stained in aldehyde fuchsin (AF), phosphotungstic acid-hematoxylin (PTAH), Azan stain (after pre-oxidation), and Victoria blue-acid fuchsin Blood and tissue samples were taken (n=10) at the predetermined intervals according to the experimental protocol The blood glucose, cholesterol, free fatty acids (FFA), organic PO_4, amino nitrogen, and liver and skeletal muscle glycogen were estimated photometrically[16] Significance of difference between the control and experimental mean values was calculated by Student's t test

Observations

Histomorphology

Pancreas is a diffused structure and its exocrine acini are scattered in the mesentery over the surface of the intestinal bulb and coils of the intestine The principal islet is in the form of a small, whitish body close to the bile duct on the surface of the intestinal bulb (Fig 1) In addition to it, a few small accessory islets are also present on the dorsal surface of the bulb The principal islet is round or oval in shape and is surrounded by connective tissue layer Two cell types, A- (= α cell) and B- (= β cell) can be distinguished in the parenchyma on the basis of selective granule staining and morphological characters (Fig 2) Both the cell types are distributed all over the islet in almost equal proportion and no spatial allocation is possible The B-cells are elongated, polyhedral in shape and possess a slightly oval nucleus lying in the middle of the cell These cells are strongly positive to AF stain (Fig 2) but are

negative to PTAH, Azan and silver stains The cytoplasm contains fine secretory granules, and the cells are commonly seen surrounding the blood capillaries throughout the islet tissue in the normal as well as control fish

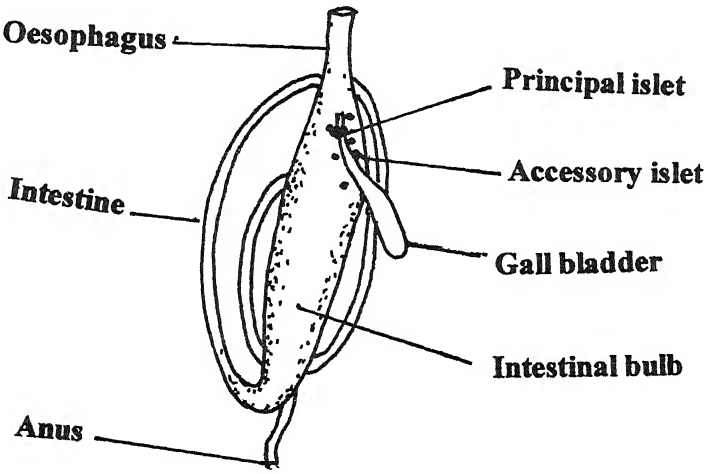

Fig 1– Dorsal view of the digestive tract of *B conchonius*, showing principal and accessory islets

The A-cells are of various shapes and size, and are scattered throughout the islet They are stained with PTAH and Azan, after preoxidation (Figs 3,4) Each cell has a round nucleus placed eccentrically When stained with Victoria blue-acid fuchsin, B-cells become blue while the A-cells are acid fuchsin positive No other cell type could be identified in the principal islet of this fish

Effect of insulin treatment on Islet cells

Fish injected with 200 I U/kg b wt insulin showed only a mild degranulation of a few B-cells during the first 2 hrs With the higher dose-(400 I U/kg b wt) a number of B-cells were depleted of granules at 2 hrs After 8 hrs of the higher dose, regressive changes in the B-cells were quite apparent (Fig 5) Cellular boundaries became indistinct and the islets showed vacuolation The islet examined after 48 hrs. of treatment showed degeneration of B-cells along with intercellular vacuoles The islets of saline injected control fish had normal A- and B- cells(Fig 6)

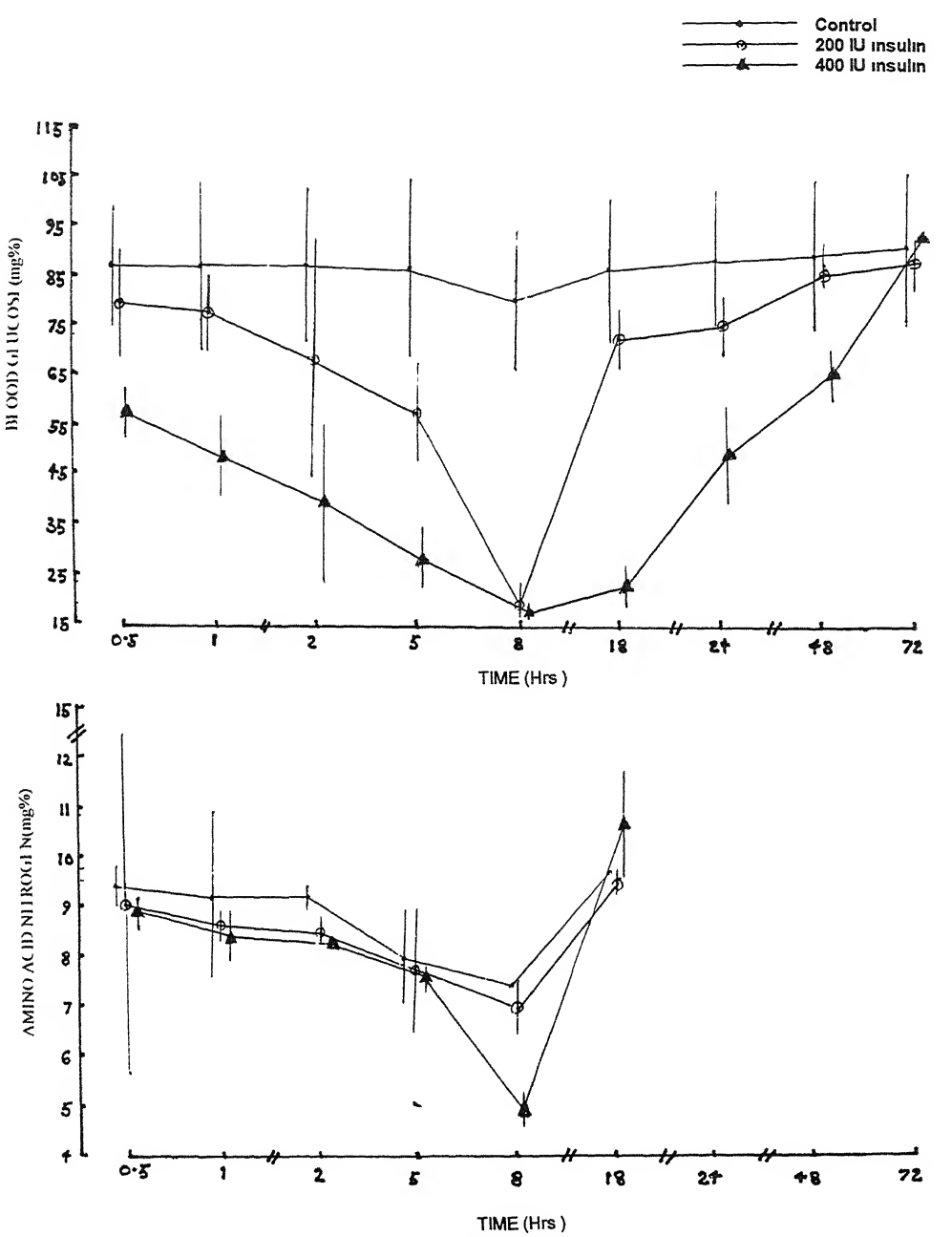

Fig 7– Effect of insulin on the blood glucose and amino nitrogen of *B conchonius*

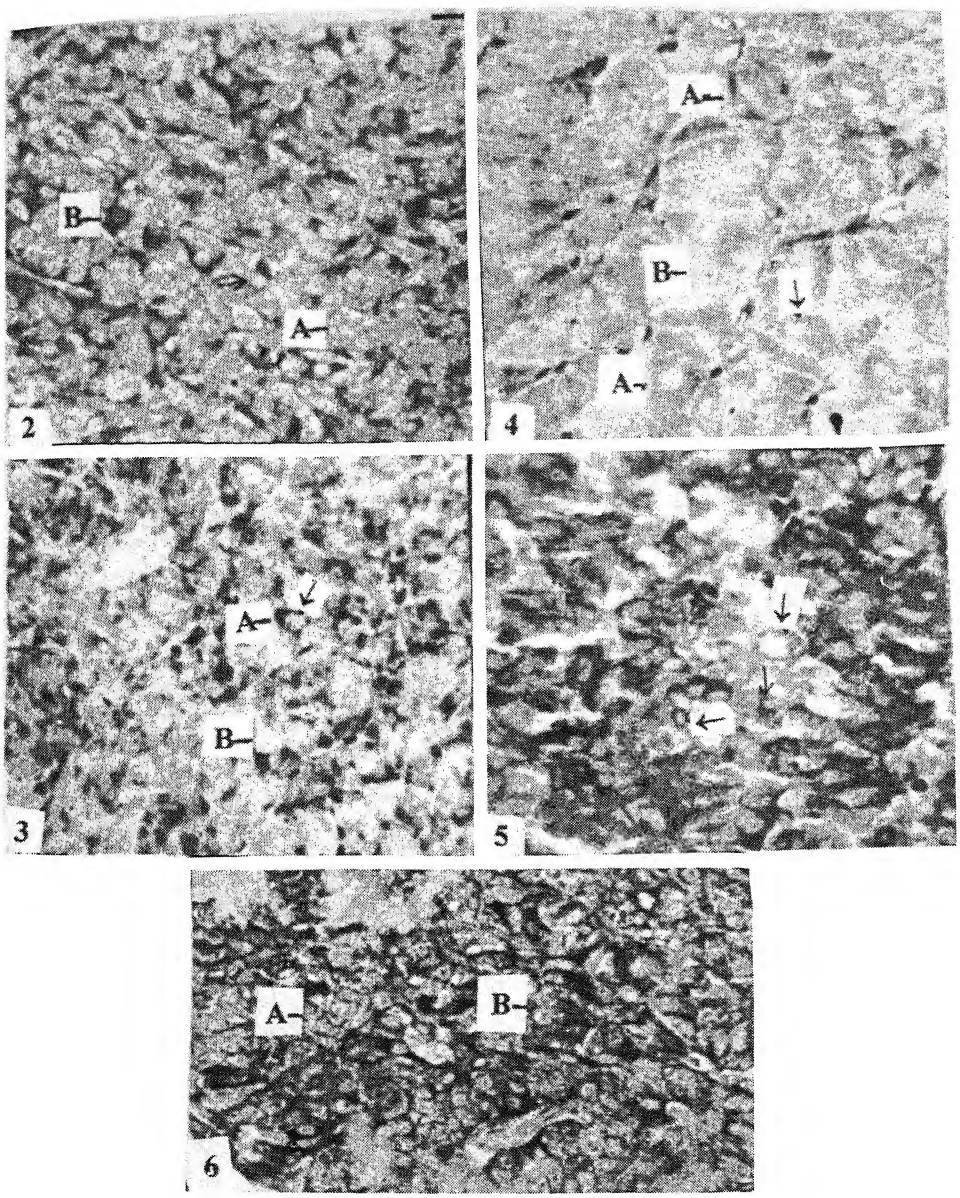

Fig. 2– Part of an islet showing AF-positive B-cell. 900X

Fig. 3– Part of an islet showing PTAH-positive A-cell. 900X

Fig. 4– Part of an islet showing azocarminophilic A-cell. OX-azan stain. 900X

Fig. 5– Islet cells after 8h of insulin (400 I U/kg b. wt.) AF-stain, showing degranulation of B-cells. 900 X

Fig. 6– Part of an islet of the control fish, showing normal A- and B- cell. AF -stain. 900 X

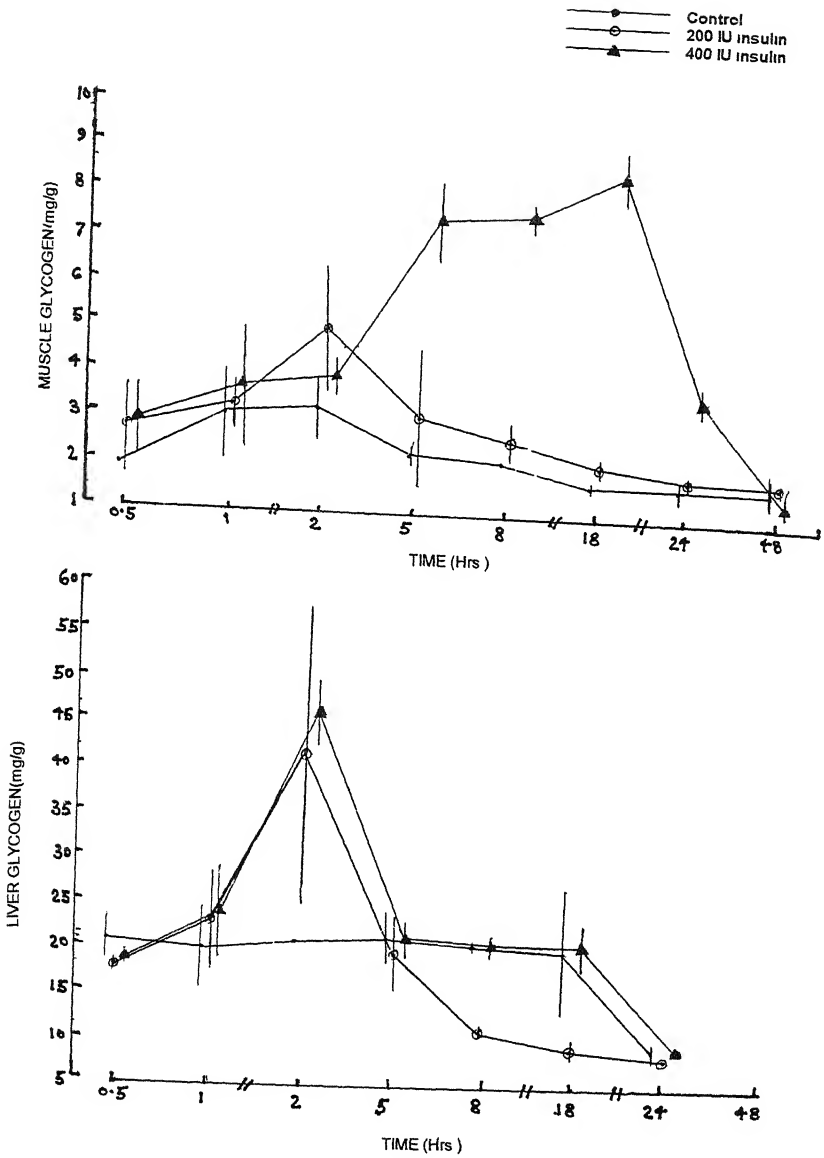

Fig 8– Effect of insulin on liver and skeletal muscle glycogen of *B conchonius*

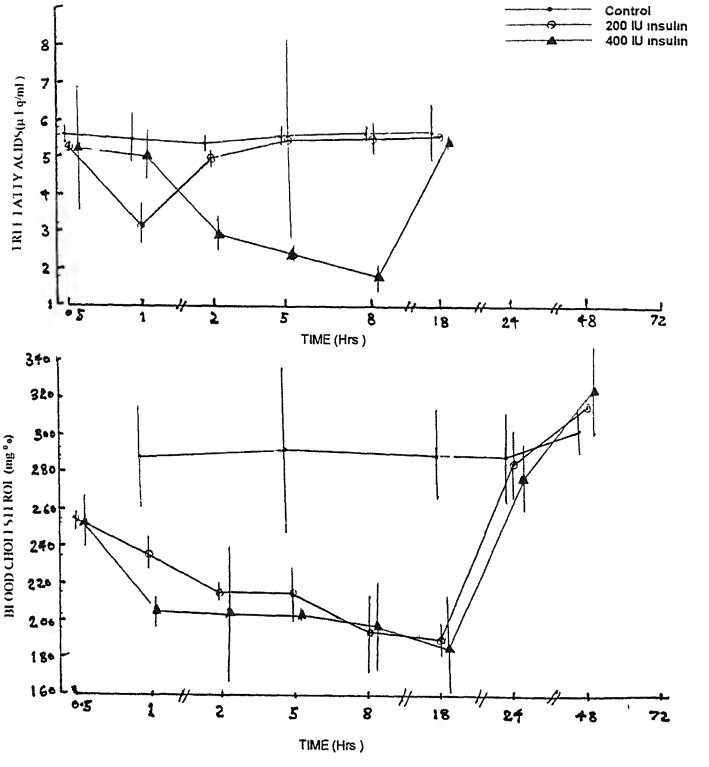

Fig 9(a)–Effect of insulin on FFA and blood cholesterol of *B conchonius*

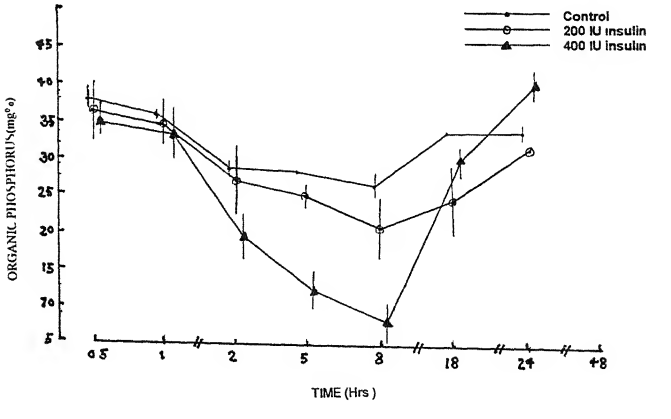

Fig 9(b)– Effect of insulin on organic PO_4 of *B conchonius*

Effect of insulin on metabolites

Insulin at lower dose evoked significant, yet recoverable, hypoglycemia beginning at 1/2 hr and the maximum reduction occurred at 8 hr post-injection ($p<0.05$). The higher dose caused significant depression ($p<0.05$) in glycemia between 5-18 hr post-insulin injection. However, euglycemic state was restored after 48-72 hrs (Fig. 7). The hypoaminoacidemia occurred at 5-8 hr after insulin injection. The higher dose elicited a significant fall ($p<0.01$) at 8 hr, followed by recovery to near control values (Fig. 7). A notable rise ($p<0.05$) in both liver and skeletal muscle glycogen was recorded following insulin treatment (Fig. 8). However, the increment was rapid but short lived in the liver, and prolonged, albeit delayed, in the skeletal muscles.

The changes in the lipid profile in the blood of insulin treated fish are depicted in Fig. 9 (a, b). Insulin caused hypocholesterolemia, which became apparent within 1/2 hr and persisted upto 18 hr post-treatment, when the drop was significant ($p<0.05$). Both the doses of insulin induced almost parallel fluctuations in the cholesterol level (Fig. 9a). The organic PO_4 levels, representing mainly the phospholipids and phosphoproteins, were slightly depressed at 1/2 hr and a gradual fall resulted in a significant reduction ($p<0.05$) 8 hr post-injection. Fish recovered from this depression towards the end of the experiment (Fig. 9b). Concomitant with the reduction in other lipid metabolites, the FFA content also decreased in response to insulin treatment. Although the lower dose failed to elicit a notable decrease the fall was significant ($p<0.01$) between 2-8 hr subsequent to administration of the higher dose. This was followed by rapid recovery at 18 hr post insulin injection (Fig. 9a). The treated fish exhibited symptoms typical of insulin shock, often coinciding with maximum hypoglycemia.

Discussion

Pancreas in *B. conchonius* is a diffused structure and its endocrine part exists as a large 'principal' islet, along with small accessory islets as reported in several teleostean species[17]. These islets have been extensively used in hormone research, and biosynthesis of somatostatin was possible using catfish islets[18]. Cytological studies on teleostean islets have been conducted by several investigators[3-6] and different cell types using different names, have been reported in various species[17]. However, some of these cells are possibly immature or developing stages of others. Studies based on immuno-chemistry and electron microscopy has yielded considerable information, and for the sake of uniformity cells of the islets are designated as A-, B-, and D- type. The agranular cells have been found to be immature precursor of granulated cells.

Moreover, all the cell types may not be found in the same islet In *B conchonius*, only two types A- and B-cells have been found in the principal islet Whether any other type of cell is present in an accessory islet of the fish needs further study

Localisation of the cell types within the principal islet varies from species to species In general, the B- and D-cells are concentrated in the central part of the islet while the A-cells are more numerous towards the periphery as in *Cottus scorpius*[2], *Gadus callarias*[19], and *Channa punctatus*[7] In *Conger japonicus*, A-cells form several layers at the periphery of the islet, followed by B-cells containing a few D-cells, and finally the central part is occupied by a fourth cell type[20] The daddy sculpin *Cottus scorpius* has two principal islets of equal size, the islet near the spleen contains A-, B- and D-cells but no F-cells, whereas the pyloric islet contains all the four cell types[21] In *B conchonius*, only one principal islet is present, and A- and B-cell types are scattered uniformly throughout the islet, with B-cells more concentrated round the blood sinusoids As regards their functional significance described in the available literature, the B-cells are known to be the source of insulin, the A-cells have been associated with glucagon secretion, while the D-cells are believed to secrete a third pancreatic hormone, somatostatin[22]

Degranulation of B-cells has been observed in some air breathing teleosts after treatment with mammalian insulin[23,24] *B conchonius* a cyprinid, also exhibited a similar response to insulin The damage to B-cells is possibly due to inhibition of endogenous insulin secretion in response to the administration of excess of exogenous hormone Many A-cells are also degranulated due to excess secretion of glucagon to suppress the effect of exogenous insulin Similar observations have been made in a catfish, *Clarias batrachus*[24] A distinct but somewhat delayed hypoglycemic action of bovine and mammalian insulins administered exogenously to some species of air breathing fishes is reported in the literature[8-12] The observations on the histology of islet cells and alterations in the metabolites of *B conchonius* as described above, suggest that this species exhibits an earlier and better response to treatment of mammalian insulin

Appearance of hypoglycemia and its duration in fishes seems to depend upon a number of factors, and some fishes are even unresponsive to high doses of insulin over a long period of time[13] *B conchonius* species also requires relatively high doses of insulin to evince hypoglycemia and one of the reason of the ineffectiveness of lower doses could be the considerable differences, both immunological and molecular, that exist between mammalian and piscine insulins, which consequently may reflect upon their biological properties Other factors, which affect insulin action in fishes are dose and route of administration, biological half life, insulin antagonists, ambient

temperature, the resting blood glucose related to mobility, and phylogenetic position of the fish[9,10,25].

Effect of insulin on glycogen stores in liver, skeletal muscles brain and myocardium is often variable and contradictory[26,27,28]. The notable rise in both liver and skeletal muscles glycogen in *B conchonius* seems to be insulin mediated and the anabolic effects of the hormone, in this respect, is clearly demonstrated. It was interesting to note a rapid but short-lived increment in liver glycogen in contrast to the rise in skeletal muscle glycogen that persisted for longer duration It is difficult, however, to draw a temporal relationship between hypoglycemia and rise in glycogen levels, since hypoglycemic nadir did not quite match with the glycogen peaks. Further, a prolonged hypoglycemia in this species can hardly be explained by a transient increase in glycogen in muscles or liver, and it is possible that this phenomenon is due to conversion of glucose into lipids.

The role of insulin in protein metabolism in fish is not completely known and the limited data that are available suggest that metabolic role of this hormone is similar to that reported in mammals[29,30] As reported in other teleosts[11], insulin caused significant reduction in amino nitrogen in *B conchonius* The hypoaminoacidemia observed in *B conchonius* may be related to uptake of plasma ·free amino acids for incorporation into proteins since protein synthesis and distribution of .free amino acids is under control of insulin in fishes[28].

In common with the responses of *Carassius auratus,* scorpion fish and the seabass to bovine insulin, *B. conchonius* also exhibited depressed FFA levels, the maximum reduction occurring at 2-8 hr post-insulin injection. However, in the pike, *Esox lucius,* bovine insulin failed to evoke any change in plasma FFA while a similar dose of codfish insulin caused an immediate and significant reduction of FFA, 1/2-9 hr after injection[31]. The organic phosphorous, representing the phospholipids and phosphoproteins, registered a decrease in *B. conchonius* paralleling the fluctuations in plasma FFA levels, and the maximal drop occurred at 8 hr post-injection in both these indices Almost similar findings have been reported in the goldfish *Carassius auratus,* subsequent to bovine insulin[32]. In *B conchonius,* however, lipid profile was notably altered following insulin injection, and as such the role of this hormone in controlling lipid metabolism, at least in this species, cannot be overlooked

Cholesterol showed significant and prolonged decrease in the species examined. Thus a situation akin to that obtaining in mammals is demonstrable in this fish Similarly in the eel, *Angulla angulla,* codfish insulin lowered plasma cholesterol level at 6 hr whilst bovine insulin was ineffective[33]. The pike also failed to exhibit

hypocholesterolemia with insulin alone but when injected with a glucose load, the plasma cholesterol fell significantly[11] Insulin, therefore, seems to exert greater effect on lipid metabolites in the presence of excess carbohydrate although possible reasons for this are unclear

The differential effects of insulins of wide phylogenetic origin are well established from studies in pike[11,31] and goldfish[32] Moreover, a variation in the ratio of cell types and consequently in the islet hormones related to age and sex have also been reported[17] The use of heterologous insulin in *B conchonius* necessitated administration of pharmacologically high doses of the hormone Thus this study shows that the response of cellular components to insulin is similar to mammals, and despite evolutionary changes in the amino acid sequence of insulin molecule in the vertebrates, the hormone promotes glucose utilization, positive nitrogen balance and lipogenesis As such, *B conchonius* can be effectively used as an experimental animal for further studies

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Intestinal protozoan parasites among Libyan and non-Libyan residents of Benghazi, Libya

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Abstract

Examination of the stools of 412 Libyan and 1783 non-Libyan nationals residing in Benghazi, Libya, during September, 1999 to August, 2000, revealed that 13 8% of the former and 15 7% of the latter contained the trophozoites and/or cysts of some of the nine intestinal protozoan parasites in them These parasites were 1) *Entamoeba histolytica,* 2) *E dispar,* 3) *E coli,* 4) *E hartmanni,* 5) *Endolimax nana* 6) *Iodamoeba butschlii,* 7) *Giardia lamblia,* 8) *Chilomastix mesnili,* and 9) *Blastocystis hominis* The proportions of positive cases between the two nationals did not show any significant difference Detailed observations were made on the single and concurrent parasitic occurrences among these nationals The importance of adopting preventive measures to control the spreading of these parasites is stressed

(**Keywords .** intestinal protozoan parasite / Libyan and non-Libyan / single and concurrent / trophozoite and cyst / stool)

Introduction

Population movement has been cited as one of the major factors for spreading the parasites worldwide[1]. The epidemiological patterns of parasite diseases in a

developing country like Libya are further complicated by the arrival of large numbers of migrant workers leading to destabilizing effects of the normal pattern of disease transmissions In order to test this hypothesis, studies were conducted during the period September, 1999 — August, 2000, on 2195 Libyan and non-Libyan nationals residing in Benghazi, Libya, and their stools were examined to detect the occurrence of trophozoites and/or cysts of intestinal protozoan parasites in them Only sporadic information[2,3,4] is available on the intestinal protozoan infection in Benghazi and these were confined on the school children of the region The objectives of the present study were to find out the occurrence of intestinal protozoan parasites among these nationals, and the percentages of positive cases among them having single or concurrent parasitic occurrences

Materials and Methods

The different nationals (age 15-45 years) whose stools were examined, were divided into 1) Libyans, who were settled for generations in Benghazi, and 2) Non-Libyans, who were residing in Benghazi for the past 3 to 5 years Majority of non-Libyans (>60%) hailed from the neighboring countries of Libya, and the rest from sub-Sahara, South and South-east Asia and East Europe Out of the total 2195 nationals whose stools were randomly selected and examined in the present study, 412 were Libyans (308 males and 104 females) and 1783 were non-Libyans (1581 males and 202 were females) Fresh stools of non-sick nationals from both groups were procured twice a week from the Central Medical Laboratory, Benghazi, in clean, numbered plastic containers Personal details including name, age, sex and nationality were recorded for each sample The stools were homogeneously mixed and directly examined on the same day of collection for trophozoites and/or cysts of protozoan parasites employing the techniques of 1) direct smear examination by the use of both normal saline solution, and Lugol's iodine solution[5], and 2) Zinc Sulphate centrifuged floatation technique[6], and were identified using the keys and descriptions[7]

Results and Discussion

The stools of 13.8% Libyans (57 out of 412) and 15 7% non-Libyans (280 out of 1783) were found to contain the trophozoites and/or cysts of one or more of nine intestinal protozoan parasites. These were 1) *Entamoeba histolytica* (cyst), 2) *E dispar* (cyst), 3) *E coli* (cyst), 4) *E hartmanni* (cyst), 5) *Endolimax nana* (cyst), 6) *Iodamoeba butschlii* (cyst), 7) *Giardia lamblia* (trophozoite and/or cyst), 8) *Chilomastix mesnili* (trophozoite), and 9) *Blastocystis hominis* (cyst) *E. histolytica* is pathogenic causing intestinal amoebiasis, whereas *E dispar* is non-pathogenic. The

cysts of *E histolytica* and *E dispar* were clubbed together as *E histolytica/E dispar* in the present study since it was difficult to differentiate the cysts of the former from those of the latter *G lamblia* was the other pathogenic parasite causing intestinal giardiasis in human beings Regarding *B hominis*, whether it is a pathogen or a commensal is still not clear All the remaining fire parasites *(E coli, E hartmanni E nana I Butschlii* and *C mesnili)* were non-pathogenics The normal tests comparing the positive cases between Libyans and non-Libyans did not show any significant difference (d = -0 05, the critical value of d = ± 1 96, sample value falls within the acceptance region)

The percentages of positive cases of Libyans and non-Libyans having single or concurrent occurrences of trophozoites and/or cysts of intestinal protozoan parasites in their stools are presented in Table 1

Rega-ding single parasitic occurrence, more than 50% of positive cases of Libyans . id non-Libyans recorded the cysts of *E histolytica/E dispar*, 12 3% Libyans and 16 8% non-Libyans the trophozoites and/or cyst of *G lamblia*, and 10 5% and 5 4% of Libyans & non-Libyans respectively had the cysts of *E nana* in their stools The percentages of Libyans having the cysts of *E coli* and the trophozoites of *C mesnili* in their stools were 3 5% and 1 8% respectively, whereas the percentages of non-Libyans having the cysts of *E hartmanni, I Butschlii, B hominis* and *E coli* in their stools ranged from 0 4% to 2 9%

Among the concurrent parasitic occurrences, 14% Libyans and 8 6% non-Libyans contained the combination of the cysts of *E histolytica / E dispar + E coli*, and 1 8% of the former and 2 1% of the latter recorded the cyst of *E histolytica / E dispar* + trophozoites and/or cysts of *G lamblia* in their stools 1 8% each of Libyans and non-Libyans recorded the cysts of *E histolytica / E dispar* + B hominis, and 1 8% of Libyans contained the cyst of *E histolytica / E dispar + E coli + E nana* in their stools The other double and triple combination of parasitic trophozoites and/or cysts were discernible in the stools of a low percentage (0 4%) of non-Libyans (Table 1)

A significant difference was not found in the percentages of positive cases between Libyans and non-Libyans (A) (F = 0 000, P>0 01) and between the percentages of these nationals having single or concurrent parasitic occurrences (B) (F = 3 55, P>0 05) of the trophozoites and/or cyst of the protozoan parasites in their stools However the interaction between A & B was found to be significantly different (F = 7 38, P<0 05)

Table 1– Percentages of positive cases of Libyan (n = 57) and non-Libyan (n = 280) nationals having single or concurrent parasitic occurrences of the trophozoites and/or cysts of intestinal protozoan parasites in their stools

Sl No	Parasites	% Positive cases in	
		Libyans	Non-Libyans
	Single parasitic occurance		
1	*Entamoeba histolytica / Entamoeba dispar*	52 6	57 9
2	*Entamoeba coli*	3 5	2 9
3	*Entamoeba hartmanni*	0 0	0 4
4	*Endolimax nana*	10 5	5 4
5	*Iodamoeba butschlii*	0 0	0 4
6	*Giardia lamblia*	12 3	16 8
7	*Chilomastix mesnili*	1 8	0 0
8	*Blastocystis hominis*	0 0	2 1
	Concurrent parasitic occurrence		
1	*E histolytica/E dispar + E coli*	14 0	8 6
2	*E histolytica/E dispar + E hartmanni*	0 0	0 4
3	*E histolytica/E dispar* + G *lamblia*	1 8	2 1
4	*E histolytica/E dispar + B hominis*	1 8	1 8
5	*E hartmanni + B hominis*	0 0	0 4
6	*E. histolytica/E dispar + E coli + E nana*	1 8	0 0
7	*E histolytica/E dispar + E coli* + G *lamblia*	0 0	0 4
8	*E histolytica/E. dispar + E nana + I butschlii*	0 0	0 4
9	*E histolytica/E dispar + E hartmanni + B hominis*	0 0	0 4

Intestinal protozoa are the etiological agents of several widespread parasitic diseases, the most common of which are caused by *E histolytica* and *G lamblia*[8] Amoebiasis is a cosmopolitan infection transmitted by the fecal-oval route, food and drink and its greatest impact is in Africa and in Asia In Africa, Egypt, Morocco and countries located between 10°N and 10°S are severely affected[9] This categorization includes Libya also Moderate to high prevalence of this parasite are reported in population residing in sub-saharan and Arabian countries also[10,11]

G lamblia the causative parasite of giardiasis was reported earlier among the school children in Benghazi[2,3,4] With the advent of AIDS there was speculation that *G lamblia* may be an important pathogen of this group, but clinical findings to date do not seem to confirm this possibility[12] Giardiasis was also reported among Jordanian[13], Egyptian[14] and Palestinian[11] populations, but is less severe in sub-saharan African countries[15]

Moderate percentages of Libyans and non-Libyans had the cyst of *E coli* in their stools High prevalence of *E coli* was reported earlier among the school children of Benghazi[3] and in Egypt[14] *E coli* is found in large intestine and is of medical importance only because it may be mistaken for *E histolytica*[7]

The most critical question concerning *B hominis* today is whether it is a pathogen or a commensal It is a common inhabitant of the human bowel and is increasingly recognized as a potential cause of diarrhea[16] Also, several reports suggest that *B hominis* may be an opportunistic infection in immuno suppressed patients with AIDS[17,18], but whether as a commensal or as a pathogen remains to be determined

The cyst of *C mesnili* were found in the stools of Libyans only, whereas the cysts of *E hartmanni* and *I butschlii* were detected among non-Libyans The recognition of non-pathogenic intestinal protozoan parasites is generally accepted as an useful epidemiological indication of the level of fecal contamination[14] The non-pathogenic forms recorded in the present study are cosmopolitan in distribution and are reported harmless in humans except for creating intestinal disturbances if found in large numbers

In conclusion, high prevalence of *E histolytica/E dispar* and *G lamblia* and their combinations, the modest prevalence of *B hominis* and its combination with other pathogenic forms in Libyan and non-Libyan residents of Benghazi, are matters of concern The gravity of the situation increases further after recent findings that *G lamblia* and *B hominis* are important and opportunistic parasites related with AIDS The possibility of transmission of some of the parasites from the alien population to the indegenous Libyans and vice versa cannot be ruled out unless proper preventive

and precautionary measures are implemented to check the spreading of these parasites These include effective environmental sanitation to prevent water and food contamination, proper sewage, adequate handling and treatment of water supplies and health education The results of the present study also indicate that more stringent parasitological surveillance of immigrant population may help in reducing the risk of transmission of infections

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Age at first delivery in Hanuman langur, *Semnopithecus entellus* around Jodhpur, Rajasthan (India)

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Abstract

Primate females begin reproduction any where between a year and 13 years of age, considering the gamut from mouse lemur to Chimpanzee The fact is that they are slower to mature than other mammals of equal body weight Timing of the onset of puberty depends on environmental factors, especially nutrition That young males and females mature faster in captivity than counter parts in wild On the basis of long field studies on Hanuman langur, *Semnopithecus entellus* at Jodhpur, Rajasthan (India), the birth rate, birth peaks, inter-birth interval and age at menarche were investigated during 1982-89 The data of three focal bisexual troops viz Kailana I, Kailana II and Bijolai obtained from 9 samples of females whose dates of birth, first menstruation, and delivery of their first baby were known This study presents that langur female reaches menarche at 26-36 months of age (average 30 0 months), conceived after variable number of cycles and age range 30 7-54 3 months (average 38 5 months) and at first delivery their age ranges from 37 3-60 9 months (average 45 2 months) Various other social and environmental factors, besides nutrition, may affects physical maturation

(**Keywords** Hanuman langur/ *S entellus*/ age / Ist delivery)

Introduction

The Hanuman langur (*Semnopithecus entellus*), a member of Indian Colobinae, perhaps is the most widely distributed of the non-human primates in Indian subcontinent It is a highly adaptable species and is still maintaining itself in various ecozones even in adverse ecological conditions in its distribution zones[1]. In the western part of its habitat, it is found predominantly organised in two types of groups viz. bisexual troops and unisexual all-male bands. The eastern fringe of the Great Indian Desert supports an isolated population of about 1800 langurs living in an area

of 150 sq kms (Rajpurohit *et al*)[2] It is an open scrub forest with undulating hillocks, orchards, gardens, crop fields and human dwellings

In the last quarter of 20th century, fairly intensive field studies on free-ranging wild population of Hanuman langur have been carried out at several locations in India, Nepal and Sri Lanka The Jodhpur population is being monitored since 1967 and has provided valuable clues to the eco-ethology and sociobiology of the species Bisexual troops contain 7-93 members (average 38 5) and in all-male bands 2-47 individuals (average 11 8)[3,4] The group numbers and locations have been depicted in Fig 1 Troops are matrilineal groups of adult females and offspring with either one adult male (unimale troops) or more than one adult male (multimale troops) The percentage of unimale troops, multimale troops, and the corresponding number of extra troop band males, varies from site to site Though, births have been observed round the year at Jodhpur but maximum births take place in one or two peaks when seasons have great variation in climate and vegetation[2]. Lancaster & Lee[5] stated that there is no general pattern among the langurs or also in macaques but it should be specific for each geographical region The estimate age at which maturity in langurs reaches is 3 to 4 years in females[6], 6 to 8 years in males[7,8,4,9,10] The gestation period is about 200 days and average cycling lengths is 24 days [6,11,12] The mean interbirth interval in langurs at Dharwar noted by Jay[7] and Sugiyama[8] is 20-24 months, at Abu is 15-30 months[13] and at Jodhpur is 8-22 months[14], 8-22 months, average 15 3 months[6], and 7.0-76 0 months, average 16.8 months[2] Maternal rejection and infantile tantrums characterizing 'weaning' occurs between 8-14 months (n=12)[15] The present paper deals to investigate the age at first birth in langurs

Materials and Methods

The present findings are part of long term (i e July 1983 to March 1987) field data on Hanuman langur, conducted around Jodhpur, Rajasthan (altitude 241 m, latitude 26° 18 N and longitude 73° 08 E) since 1980. In and around Jodhpur the climate is arid and characterized by uncertain and variable rains (annual mean rainfall ca 360 mm) and wide range of temperature (maximum up to 50° C and minimum below 0°C). The Hanuman langur groups here are distributed over a 30 km diagonal ridge running from the village Arna in the west to Daijar in the northeast passing through Jodhpur Fort (*see* Fig 1) There are no other langur troops within a radius of 100 km. Water is available throughout year for all the groups through artificial lakes, tanks and ponds Some of these groups dwell close to human habitations or abandoned building and people regularly feed most of the groups[13]

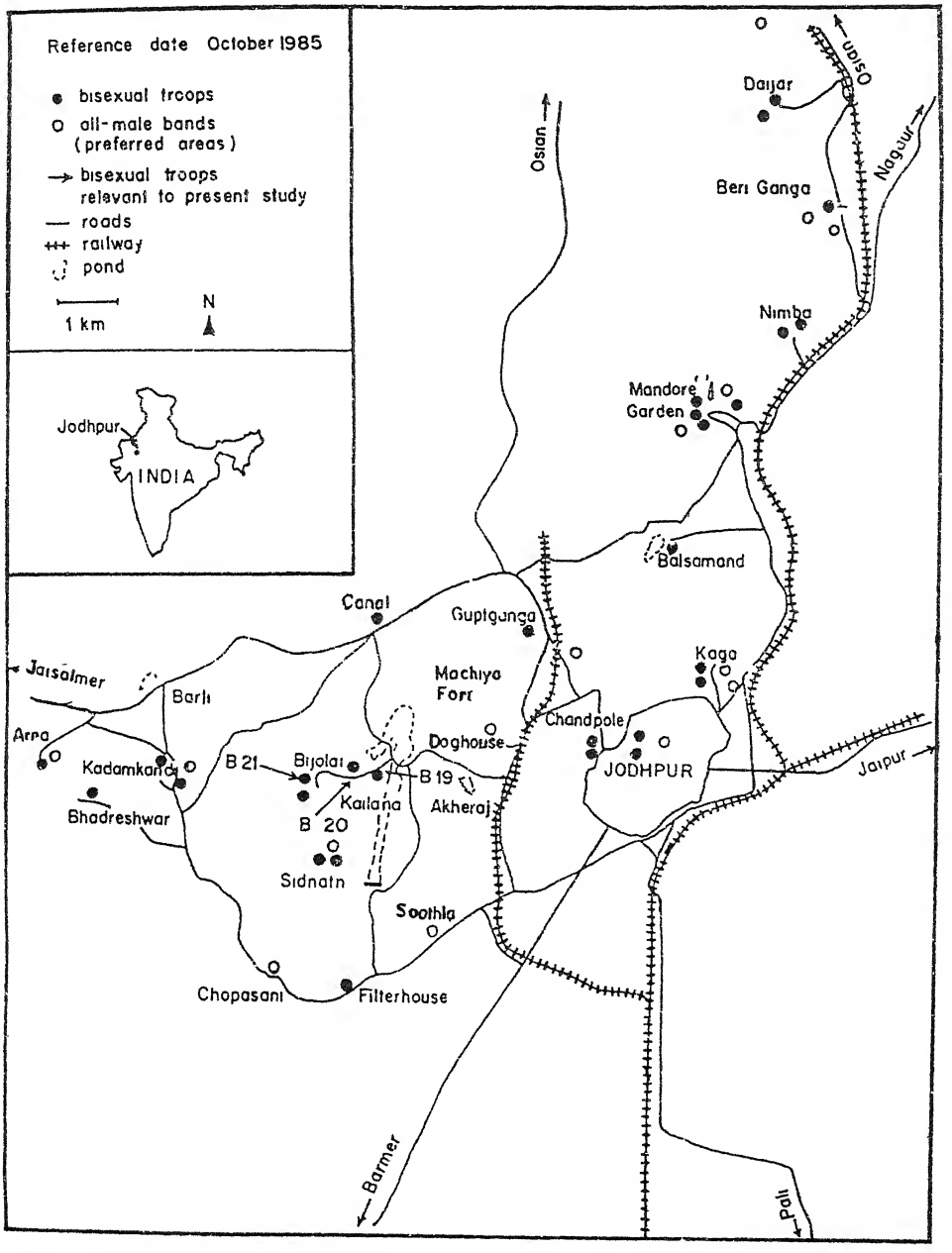

Fig 1– Location of bisexual troops and all-male bands around Jodhpur

The focal troops *viz* Kailana I (B 19), Kailana II (B20) and Bijolai (B21) provided nine samples for menarche study in the langurs (during 1980-1987) Before the commencement of a particular study all the adult individuals of these focal troops were individually identified and every social, demographic and behavioural changes were recorded with the help of *ad libitum,* scan and focal animal sampling[16] Jodhpur langurs are not too shy and are easily observable at ground for maximum hours of a day Except some feral dogs, they do not have any natural predator

Results

Study on three focal troops provided nine samples of female langurs, whose birth dates and the dates when they delivered their first infant are known The birth dates and age at first delivery were recorded in long-term observation of 3 neighbouring bisexual troops (i e B19, B20 and B21) with individually known females whose menstrual cycles (as menstrual bleedings are clearly visible in this species here) were continuously monitored It is unlikely that any birth in focal troop during study period has missed because all the adult females were individually identified and regularly followed for their reproductive success etc In this study all females reached their menarche at 26-36 months of age (average 30 0 months) and conceived after variable number of cycles, at the age of 37 7-54 3 months (average 38 5 months) The age, at which langur females have delivered their first baby, ranges between 37 3 and 60 9 months, average 45 2 months (cf Table 1) Of these nine cases in seven the first baby was female, in one case it was male and the sex of remaining one could not be ascertained, before it disappeared after birth The sample was not having sufficient number of male infants on first birth to compare the age of mother at first delivery of male versus female infant

Discussion

The number of children born by a female can be described by the age at which she begins reproduction, the age at which she stops reproducing and the inter-birth interval Primate females begin to reproduce some where between one year and 13 years of age, considering the gamut from mouse lemur to chimpanzee The significant fact is that they are slower to mature than other mammals of equal body weight The majority of monkeys, and even the larger lemurs, do not bear offspring before three years of age[17]. The present study suggest that langur female reach menarche at about 30.0 months, which nearly supports Winkler *et al* [6] in which they have stated this age about 29 0 months But then after variable number of cycles females conceived and delivered their first baby at the age of about 45 2 months ranging between 37 3-60 9

months (n=9-present study), while Winkler *et al*[6] and Sugiyama[8] noted this age about 40 months

Table 1– *Semnopithecus entellus,* age of female langurs at first birth (in months)

Animal No (F=female)	Troop No (B=bisexual)	Birth Date (A)	Date of first delivery (sex of infant) (B)	Age at first birth (B-A)
F 4 4	B19 (Kailana I)	08 Feb 80	02 Jul 83 (F)[a & b]	40 8
F 6 3	B 19 (Kailana I)	26 Dec 79	08 Aug 83 (F)[a]	43 4
F 2 3	B 19 (Kailana I)	03 Aug 80	09 Sep 83 (F)[a]	37 3 (Min)
F 2 4	B 21 (Bijolai)	06 Jul 80	29 Feb 84 (M)[a & b]	43 8
F 3 2	B 19 (Kailana I)	04 Aug 80	16 Mar 85 (F)[d & a]	55 4
F 13	B 20 (Kailana II)	Mar 82	03 May 85 (?)[b & a]	39 0
F 6 4	B 21 (Bijolai)	15 Sep 81	01 July 85 (F)[b & c]	45 6
F 14	B 20 (Kailana II)	15 Jun 82	10 Oct 85 (F)[c & a]	40 3
B 2 5	B 21 (Bijolai)	15 Feb 82	12 Mar 87 (F)[c & a]	60 9 (Max)
Mean				45 2 (n=9)

Observers a= L S Rajpurohit, b= G Agoramoorthy; c= A Srivastava and d= C Borries

Hadidian & Bernstein[18] have investigated the median age at first delivery for seven pigtails *(Macaca nemestrina)* is 47 months, ranging 43 - 67 and for ten rhesus *(Macaca mulatta)* is 43 months, with a range of 36 - 44 months The mean age at first delivery has been given as 55 months in Japanese macaques *(M fascatta fascatta)* and 46 months in crab eating macaques (*M fascicularis*)[19] Timing of the onset of puberty depends on environmental factors, especially nutrition In the semiarid area like Jodhpur, natural food is not always in plenty and therefore such situation is responsible for delayed first delivery of baby in langurs here In many of zoos and captivities, it was found that young males and females mature earlier than their wild counter part[20] This is in accordance with the more extensive human data There is controversy as to how much chronic malnutrition reduces fertility. However, the data unequivocally indicate that well-nourished populations of girls may reach menarche at 11 to 13 years, as in present day in Europe and America Various others social factors, besides nutrition also affect the physical maturation

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Impact of physiological condition of fifth instar larvae of *Antheraea mylitta* on rate of feeding and assimilation and its nutritional requirements

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Abstract

Larval nutrition gets affected with the change in physiological condition of the larvae Mean daily food ingested, assimilated, feeding rate and assimilation rate tend to decline significantly in the fifth instar larvae of *Antheraea mylitta* under different physiological conditions, like daily food deprivation for 4 hr (PC_1), 8 hr (PC_2), 12 hr (PC_3) and parasitization with uzifly (PC_4) and *Nosema* sp (PC_5) over control (PCo) The decline in gain in body weight (by 20 5% to 41 9%) and growth rate (by 15 1% to 37 7%) were found significant over the uninfected control Daily mean fresh body weight gain by the larvae were recorded as 1 2 g in the PCo while the same were recorded as 0 87g, 0 73g, 0 54g, 0 75g and 0 65g in PC_1, PC_2, PC_3, PC_4 and PC_5 respectively Survival rate of the larvae declined significantly (by 14 2% to 55 6%) and the instar duration got extended by 2 to 6 6 days under physiological stress conditions Nutritional requirement for unit growth and cocoon shell formation also got affected in the larvae

(**Keywords** *Antheraea mylitta / larvae* / V instar / physiological condition / nutritional requirement)

Introduction

The quantitative nutritional aspect of insect nutrition invited greater attention after the extensive review made by Waldbauer[1], although Hiratsuka[2] had started the quantitative studies for silkworm, *Bombys mori* much earlier The quantitative consumption and utilization of food are essential for growth, development and reproduction The active and dynamic process of larval feeding under the influence of quality and quantity of food has an immense effect on growth rate, developmental time, final body weight, survival and reproductive potential[3] Adverse effects of

restricted feeding and parasitic infection on food utilization parameters and growth in insects have been reported[3-15]

Antheraea mylitta is a wild sericigenous insect of India and is polyphagous The larva is a continuous feeder, consumes and utilizes sufficient food for its growth and accumulates sufficient energy reserve for its non-feeding stage The feeding and food utilization in the larvae is often influenced by its physiological condition Although some authors had conducted quantitative nutrition studies in *A mylitta*[16-18], no report is available on the rate of feeding and assimilation in the larvae and food requirement for its growth and cocoon shell formation when the larvae suffer from different physiological stress condition, which is the aim of the present study

Materials and Methods

Antheraea mylitta Drury (Daba Bivoltine) (Lepidoptera Saturniidae) was chosen for the study because of its high exploitation potency In the experiments V instar larvae were used because maximum food ingestion (78 to 81 %) and assimilation (73 to 77%) were recorded during this instar[16] where the impact of physiological stress condition of the larvae can be studied properly Leaves of *Terminalia tomentosa* was given as larval food as it supports the growth better[16,19] Six physiological conditions were studied .

PC_0 = Control (healthy and uninfected fifth instar larvae), continuous feeding

PC_1, PC_2 and PC_3= Healthy and uninfected fifth instar larvae subjected to 4, 8, 12 hr food deprivation daily respectively

PC_4 (Parasitization by uzifly) = Healthy and uninfected fifth instar larvae were parasitized by uzifly *(Blepharipa zebina* Walker, Diptera Tachinidae) on the first day as described earlier (Rath *et al* [15]) The level of parasitization was maintained at 5 eggs per larvae

PC_5 (Infected with microsporidia, *Nosema sp)*= *Nosema* infected fifth instar larvae (10 650 x 10^5 spores/ml)

Larvae were fed with sufficient quantity of pre weighed leaves of *T tomentosa* twice a day[16] There were 5 replications with 20 larvae in each Buffer stocks were maintained to compensate mortality The mature larvae were allowed to spin the cocoon out door after completion of feeding treatment The shell content was weighed

after pupation Data recorded were based on dry weight basis Food intake, assimilation, relative growth rate (RGR) were measured following Waldbauer[1] Rate of feeding and assimilation were measured following Delvi and Pandian[20] The middle body weight of an individual (calculated by adding half the mean net growth to the initial mean body weight) was used for calculation of rate of feeding and assimilation The results were analysed for significance using ANOVA Study was undertaken during October-November in prevailing climatic conditions (temp 22-24° C and 77-80% RH)

Results and Discussion

Growth, developmental time and survivability of larva Absolute growth and relative growth rate (RGR) of the larva significantly decreased when the physiological condition of the larva was disturbed (Table 1) The decline in weight gain and RGR was in the order of $PC_3 > PC_5 > PC_2 > PC_4 > PC_1$ The highest and the lowest recorded decline in weight gain were 41 9% and 20 5% respectively, whereas, the highest and the lowest decline in RGR recorded to be 37 7% and 15 1% respectively The larvae under physiological stress took more time to attain maximum weight following lower RGR While the larvae in control condition (PC_0) attained maximum weight on 18th day, larvae in different physiological conditions (in PC_1, PC_2, PC_3, PC_4 and PC_5) attained maximum weight on 20th, 22nd, 23rd, 21st and 21st day of the instar respectively The absolute body weight did not differ significantly up to 6th day, but thereafter it declined significantly over control in all physiological conditions studied From the study it is revealed that prolongation of feeding period become necessary when the larvae are either under short supply of food due to starvation or attacked by parasites to meet their nutritional requirement to maintain a nutrient reserve for non-feeding stages of the insect Studies in insects including sericigenous species have been reported similar findings[6,7,12-15,21] Survivability of the larvae declined significantly in different conditions, the lowest survivability was observed in PC_3 where only 30% larvae survived against the control (PC_0) value of 67 6% Food deprivation in *B mori* (6 hr/day) results 37 2% decline in survivability over control[13], which confirms our findings

Rate of feeding and assimilation· During the entire V instar duration under different physiological conditions of larvae the dry matter ingestion and assimilation declined significantly to the tune of 11 3% to 25 7% and 12 7% to 40 3% respectively over control (Table 1), The daily dry matter ingestion declined to the tune of 19 1%, 32 1 %, 41 3 %, 32 5 % and 43 7% and assimilation by 29 2%, 43 8%, 54 2%, 31 3% and 35 4% in PC_1, PC_2, PC_3, PC_4 and PC_5 respectively

Table 1– Effect of physiological condition of fifth instar larva of *Antheraea mylitta* on developmental time, survivability, rate of feeding and assimilation and food requirements (dry weight basis)

Parameters	Physiological conditions of larva						CD at
	PC_0	PC_1	PC_2	PC_3	PC_4	PC_5	5%
Instar duration (days)	20 8	22 8 (+9 62%)	25 4 (+22 12%)	26 8 (+28 85%)	26 0 (+25 00%)	27 4 (+31 73%)	1 710
Survivability (%)	67 6	58 0 (–14 20%)	47 0 (–30 47%)	30 0 (–55 62%)	53 2 (–21 30%)	47 4 (–29 88%)	5 672
Mean total food ingested (g)	52 44	46 51 (–11 31%)	43 46 (–17 12%)	39 55 (–24 58%)	44 65 (–14 86%)	38 98 (–25 67%)	2 495
Mean total food assimilated (g)	19 87	15 60 (–21 49%)	13 82 (–30 45%)	11 87 (–40 26%)	17 35 (–12 68%)	16 85 (–15 20%)	1 388
Mean daily food ingested (g)	2 52	2 04 (–19 05%)	1 71 (–32 14%)	1 48 (–41 27%)	1 70 (–32 54%)	1 42 (–43 65%)	0 146
Mean daily food assimilated (g)	0 96	0 68 (–29 17%)	0 54 (–43 75%)	0 44 (–54 17%)	0 66 (–31 25%)	0 62 (–35 42%)	0 092
Feeding rate (g/ g body wt / day)	0 43	0 39 (–9 30%)	0 34 (–20 93%)	0 33 (–23 26%)	0 33 (–23 26%)	0 29 (–32 56%)	0 025
Assimilation rate (g/ g body wt / day)	0 16	0 13 (–18 75%)	0 11 (–31 25%)	0 10 (–37 50%)	0 13 (–18 75%)	0 13 (–25 00%)	0 013
Gain in body weight (g)	6 49	5 16 (–20 49%)	4 81 (–25 89%)	3 77 (–41 91%)	5 10 (–21 42%)	4 61 (–28 97%)	0 417
RGR	0 053	0.045 (–15 09%)	0 039 (–26 42%)	0 033 (–37 74%)	0 040 (–24 53%)	0 035 (–33 96%)	0 004
Shell wt (g)	2 14	1 82 (–14 95%)	1 24 (–42 06%)	1.00 (–53.27%)	1 38 (–35 51%)	1 55 (–27 57%)	0 122

Table 1 Contd

Table 1 Contd

Ingesta/ g body wt	8 09	9 01 (+11 37%)	9 04 (+11 74%)	10 50 (+29 79%)	8 76 (+8 28%)	8 46 (+4 57%)	0 840
Digesta/ g body wt	3 07	3 02 (–1 63%)	2 87 (–6 51%)	3 15 (+2 61%)	3 40 (+10 75%)	3 66 (+19 22%)	0 281
Ingesta/ g shell	24 54	25 72 (+4 80%)	35 13 (+43 15%)	39 67 (+61 65%)	32 35 (+31 82%)	25 09 (+2 24%)	2 758
Digesta/ g shell	9 30	8 58 (–7 74%)	11 17 (+20 11%)	11 91 (+28 06%)	12 57 (+35 16%)	10 84 (+16 56%)	1 068

The rate of feeding and assimilation declined significantly in the larvae, which suffered from different physiological conditions Food ingestion, assimilation and utilization got affected when restricted feeding and parasitic attack were observed[3-15,22] Decline in the rate of feeding followed by decline in the rate of assimilation due to various physiological conditions resulted in reduced growth rate Food consumption and digestibility were affected in *B mori* upon parasitization by uzi fly[12,23] so also in *A mylitta*[15] Growth inhibition was might be due to host parasitic interaction leading to metabolic changes in the host as reported in *B mori* and other insects[24-26] Food utilization efficiency was found affected following parasitization[12,15,27] Food utilization parameters got derange due to microsporidia infection was already reported in other insects[11,14] All these corroborate our present findings

Silk Production Silk production (expressed as shell weight) in *A mylitta* declined significantly in all experimental conditions The decline level were in the order of PC_3 (53 3%) > PC_2(42 1%) > PC_4 (35 5%) > PC_5 (27 6%) >PC_1 (14 9%) (Table 1) Increase in the period of food deprivation as in PC_1, PC_2 and PC_3 decreased the shell content in *A mylitta* further confirmed the findings observed in *B mori*[7,13] The lower food intake followed by lower assimilation in the larvae under different physiological conditions ultimately affected the silk production[7,12,13,15] This might be due to the depletion in amino acid pool which could be attributed to lower rate of ingestion and assimilation following change in physiological conditions of the larvae thereby amino acid flow to the silk gland could not be maintained which hampered the silk synthesis Amino acid content declined in larvae infected with *Nosema* and uzi fly in insects including *B mori* and *A. mylitta* further confirmed our findings[28-30].

Food requirement for growth and shell production Food requirement for unit growth and shell production got affected following change in the physiological status of the larvae Dry ingesta required per gram body weight increased over control in all physiological conditions studied (by 4 6% to 29 8%) but was only found significant in PC_1 , PC_2 and PC_3 (Table 1) On the contrary, digesta requirement per gram body weight increase were found significant in PC_4 and PC_5 Dry ingesta requirement per gram shell production increased significantly in PC_2, PC_3 and PC_4, but digesta requirement increased significantly in all physiological conditions studied except PC_1 where it declined by 7 7%, The increase recorded in digesta requirement ranged from 16 6% to 35 2% Increased food requirement for growth and shell formation might be due to diversion of considerable amount of food material towards maintenance of life activities

Thus, the above physiological condition led to a decline in food intake followed by assimilation and with the decrease in the rate of feeding and assimilation resulted in decline in growth rate and overall gain in body weight leading to poor silk production The physiological conditions also limit the survivability of the larvae In the present study it was found that the damage caused due to disturbed physiological conditions of the larvae were in the order of $PC_3 > PC_5 > PC_2 > PC_4 > PC_1$ Therefore, it could be concluded that utmost care should be taken during the rearing to retain the normal physiology of the larva to increase the productivity

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Development of indigenous nucleic acid probe for the detection of *Potato virus Y*

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Abstract

A common Indian strain of *Potato virus Y* (PVY°) was characterized by PCR amplification of 5' UTR and P1 protein stretching about 969 nucleotides from 5' proximal part of the genome The amplified PCR product was gel purified and cloned into pGEMT easy vector The suitable clone was picked up and confirmed by restriction digestion. The plasmid was linearised and *in vitro* transcripts were labeled with α^{32} P-UTP and a high rate of incorporation was observed PVY detection was achieved through autoradiography without any cross-reaction with other common viruses such as *Potato virus X* (PYX), *Potato virus S* (PVS) and *Potato leafroll virus* (PLRV) The signal was observed upto a dilution of 1 1024 This indigenously developed probe was found to be highly effective and sensitive for detection of PVY by comparative simultaneous probing with the plasmid received from CIP Lima, Peru Various field/ germplasm and glasshouse samples from potato, tobacco and datura with mosaic/mottle and veinal necrosis were tested

(**Keywords** PVY/ nucleic acid probe/ NASH/ P1 protein)

Introduction

Potato virus Y (PVY) the type species of the genus *Potyvirus* of the family *Potyviridae*[1], has a world wide distribution and is one of the most economically important viruses in potato All members of the family comprise flexuous filamentous particles containing a monopartite single-stranded positive sense RNA genome of ~ 9700 bases long with a small protein (VPg) covalently linked to the 5' terminal nucleotide and a poly (A) tail at the 3' terminus[2] PVY isolates from potato have been traditionally classified into three strains[3,4] Y^O, Y^N and Y^C Yield losses of 10-80 % depending upon the strain, cultivar and time of infection have been reported[5,6]

Various laboratories have applied nucleic acid spot hybridization (NASH) technique to detect potato viruses and have reported that its sensitivity of detection is

several folds higher than ELISA[7,8,9] For inspection and certification, rapid and sensitive tests for virus detection and identification are required Development of nucleic acid hybridization technique has resulted in extremely reliable and sensitive diagnostic procedure for the detection of infectious agents due to sequence similarity provided by the recognition of complementary nucleic acid strands[10] Therefore, efforts were made to develop a nucleic acid probe to ensure sensitive and reliable detection of PVY

Materials and Methods

A Extraction of RNA

PVY was purified from *Nicotiana glutinosa* two weeks after inoculation following the modified protocol of Khurana *et al* [11] Coat protein from the purified virus was removed by the addition of 5 µl Proteinase K (5mg/ ml) and 2 µl SDS (10%) in a 200 µl reaction volume This was followed by incubation at 37°C for 30 minutes and subsequent phenol chloroform extraction RNA was precipitated with 2 5 volumes of ethanol and 1/10 of sodium acetate (pH 5 2) Total plant RNA was also extracted using RNeasy Plant Mini kit (Qiagen) and subsequently used for PCR amplification The RNA concentration was estimated spectrophotometrically

B cDNA Synthesis and PCR Amplification

cDNA was synthesized using reverse transcription system (Promega) as described by the manufacturer The reverse transcription mixture had a final concentration of reaction components- Ix Reverse Transcription buffer (10 mM Tris-HCl, 50 mM KCl, 0 1% Triton® X-100), 5 mM $MgCl_2$, 1 mM of deoxyribonucleotides (dNTPs) mix, 20 units of RNasin, 600 ng of specific downstream primer d (Table 1), 1 µg (4 µl) viral RNA (which was first denatured and chilled on ice) and 15 U of AMY reverse transcriptase All were added to a 20 µl total reaction volume The mixture was then incubated at 42°C for 1 hour for RT and subsequently incubated at 95°C for 2 minutes to terminate the RT reaction The total volume was made up to 100µl by adding 80 µl nuclease free water to the cDNA

PCR was performed in 3 tubes containing 5 µl cDNA per 25 µl of the reaction solution having 1 25 µl of 2 mM dNTP's, 2 5 µl of 10X PCR buffer (Perkin Elmer) (100 mM Tris HCl, 500 mM KCl, 15 mM $MgCl_2$, 0 01%(w/v) gelatin), 0 30 µl (10U/µl) of Amplitaq DNA polymerase (Perkin Elmer), 0.50 µl(600 ng/µl) of each primer a-d in tube no 1, b-d in tube no 2 and c-d in tube no 3 respectively Cycling

conditions were optimized to an initial denaturation at 94°C for 5 min and 40 subsequent cycles of 1 min 94°C, 1 min 55°C, 1 min 72°C and, the final extension at 72°C for 10 min Following amplification, 10µl PCR product was run on a 0 8% agarose gel containing 0 5 µg/ml ethidium bromide One Kb DNA ladder (MEI Fermentas) was used to determine the size of the amplified product

Table 1– Primers used for the PCR amplification of the PVY

PRIMER	SEQUENCE 5'-3"	Fragment (bp)
a	AACACTCACAAAAGCTTTCA	928
d	TG(C/T)GA(C/T/A)CCACGCACTATGAA	
b	T/(C/T)A(C/T)AAAC(A/G)CT(C/T)ATT)ATT(C/T)TCAC	923
d	TG(C/T)GA(C/T/A)CCACGCACTATGAA	
c	AATTAAAACAACTCAATACA	969
d	TG(C/T)GA(C/T/A)CCACGCACTATGAA	

C Cloning of the amplified product

The amplified fragment obtained with the (c-d) combination was eluted from the gel using Clean Gene1 kit (Bangalore Gene1) and AT cloned in pGEM- T Easy vector (Promega) The recombinant plasmid was named pKM/YV(Y) The authenticity of the cloned gene was confirmed upon restriction release of the fragment with *Eco RI* and *Not I*

D Preparation of 32 P-labeled probes

The cloned plasmid pKM/YV(Y) containing the cDNA insert of PVY was first linearized with *Sac II* restriction enzyme. *In-vitro* transcription was carried out using RiboProbe® In Vitro Transcription System (promega) following a modification of the procedure described by Melton *et al* [12] The transcription mixture, containing 1 µg of linearized plasmid template, 0 5 mM each unlabeled nucleoside triphosphates, 70 µCi α^{32} P-UTP in the presence of 1 unit per microlitre of ribonuclease inhibitor, was incubated with 20 units of SP6 RNA polymerase for 120 min. at 38°C. The mixture was then incubated at 37°C for 20 min with 1 µl RQl DNAse (1 unit/µl) to remove

the DNA template Phenol/chloroform-extracted RNA was ethanol precipitated and resuspended in TE buffer The run-way- transcripts were obtained upon labeling

E Extraction and spotting of samples

Leaves were homogenized in formaldehyde (37%) and 10X SSC (1ml/g of tissue) and the homogenate was centrifuged at 7000K for 5 min Five microlitre of the aqueous phase of each sample was spotted onto the nitrocellulose membrane pretreated with distilled water and 20x SSC

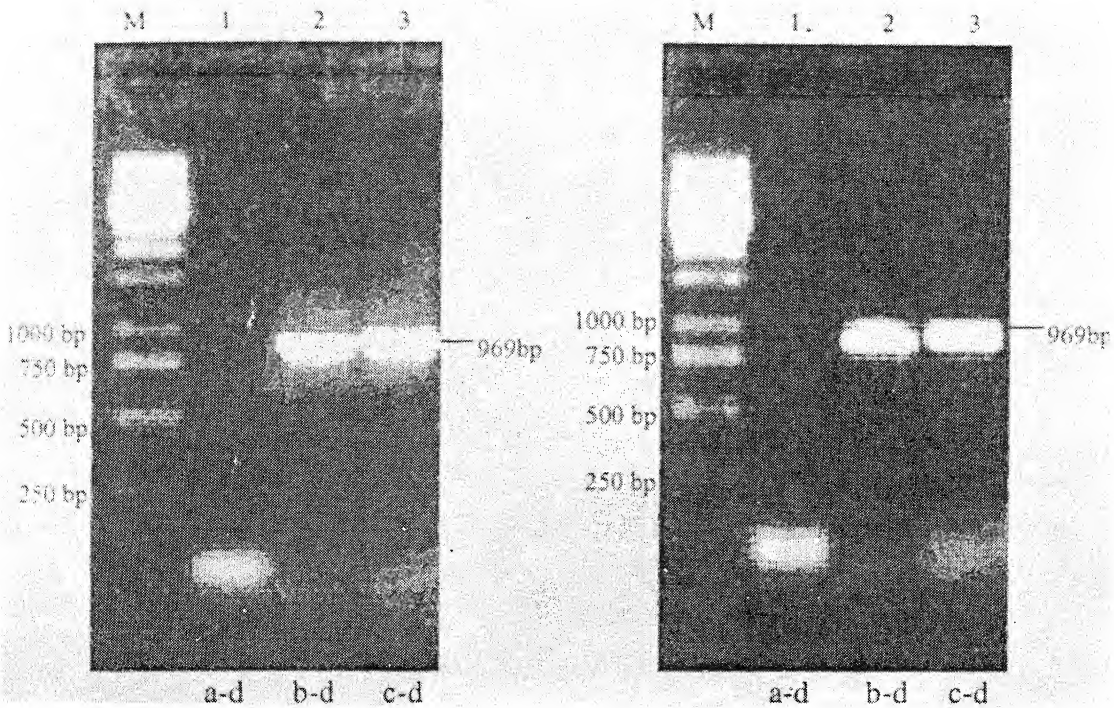
F Hybridization with the ^{32}P-labeled RNA probes

The NCM's were placed in hybridization bottle containing 9 6ml hybridization buffer [De-ionised Formamide (50%), sodium cacodilate (25 mM) and SDS (0 125%)] and incubated for 10 minutes at 55°C The membranes were blocked by adding 1ml of denatured calf thymus DNA (6 mg/ml) followed by incubation at 55°C for 10 min The next incubation was done with 2 4 ml of 50% dextran sulphate at 55°C for 10 min The probe was then added in each tube and hybridized overnight at 45°C The washing was done initially with wash buffer 1[0 36 M NaCl (21g/L), 20mM Tris base(2 4 g/L), Conc HCl (1 48 ml/L) and 20% SDS (5 ml/L)] twice for 20 minutes at room temperature The second washing was performed with wash buffer 2 [0 1 X SSC and 0 1 % SDS (l 1)] at 65°C for 30 minutes and twice in wash buffer 3 [2X SSC] for 10 minutes at room temperature Washed once again for 20 minutes in 100 ml washing buffer 3 with 20 µl RNAse (stock: 10 mg/ml) at room temperature and finally, once again in washing buffer 2 for 20 minutes at 50°C The membranes were dried at room temperature and detection was carried out by autoradiography at –70°C for overnight using Kodak X-ray films

Results and Discussion

The virus purified for production of antisera and total RNA extracted using commercial kit resulted in bands of same intensity, suggesting that anyone method, based on the feasibility and availability of chemicals may be used for PCR amplification

Accurate identification of specific strains and determination of their genetic variation is important in designing a molecular detection strategy The existence of sequence or symptom variants pose a requirement of separate sets of primers or probes The P1 protein is the least conserved protein among the potyviruses and the



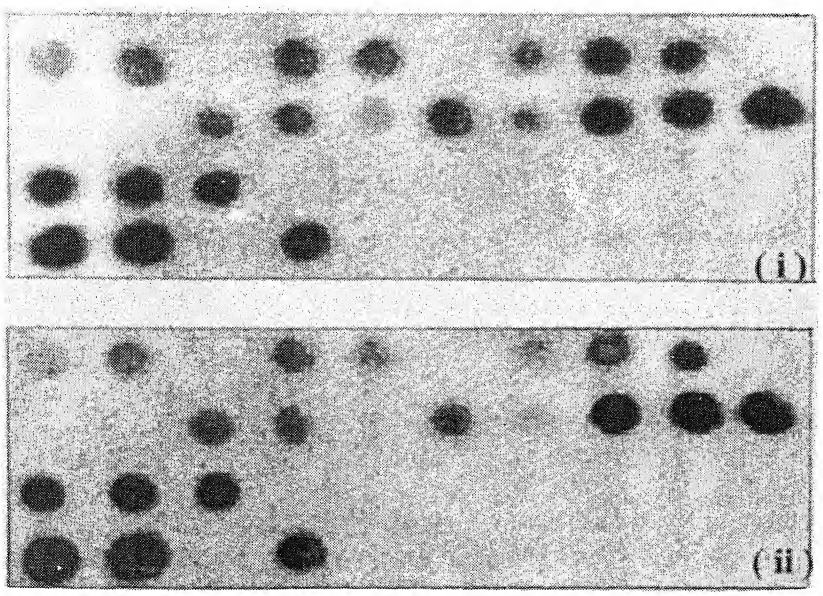


Fig. 1– (a,b) PCR amplification of the genomic segment of PVY

Lane M: 1Kb ladder (MBI Fermentas), Lane 1 : PCR set-up with primer pair a-d (For PVY^N) : No band observed, Lane 2: Amplification with primer pair b-d (Amplifying PVY°), Lane 3: Amplification with primer pair c-d, for amplifying all PVY strains.

Fig. 2– Comparative detection of PVY infected samples

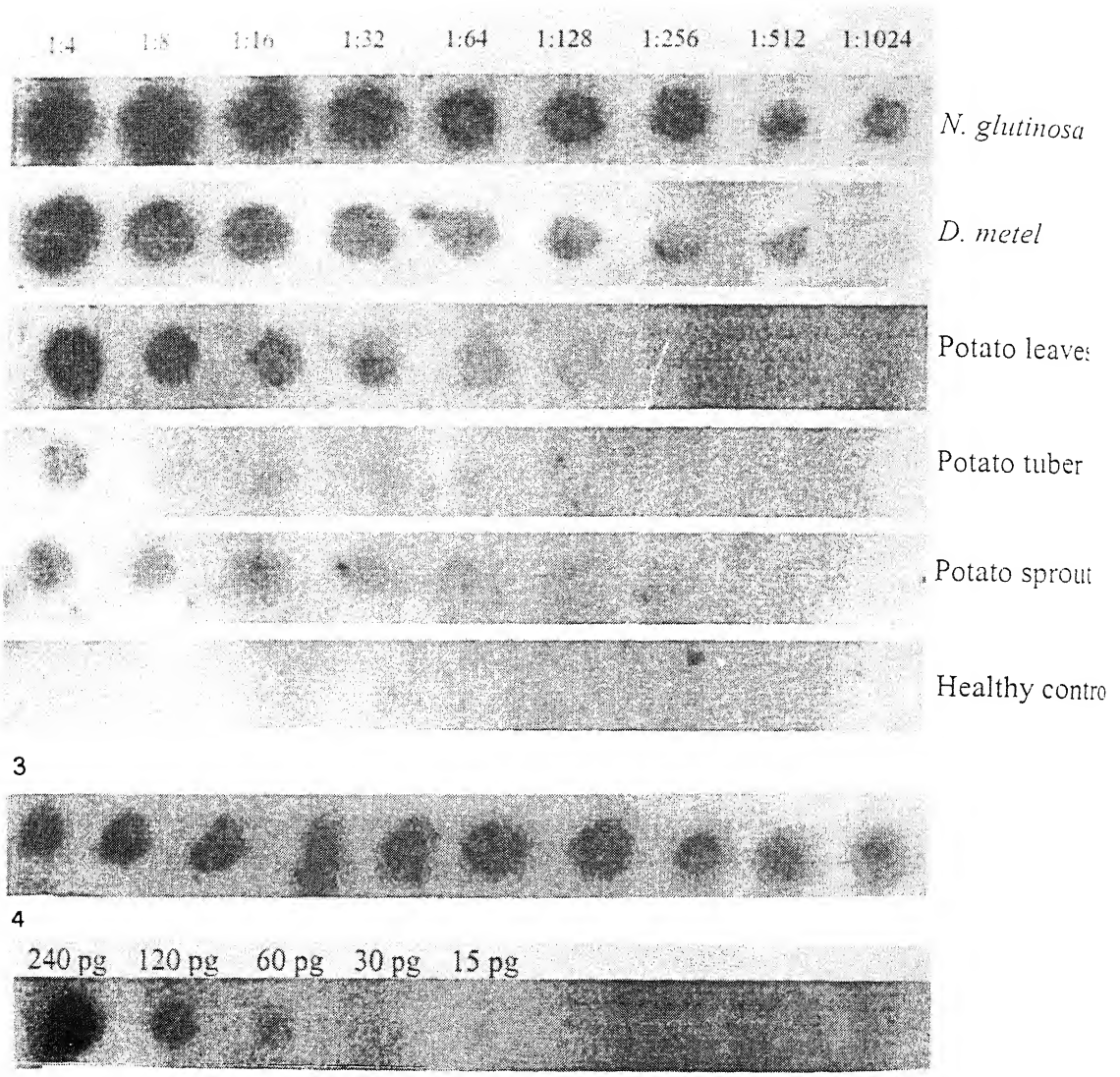


Fig. 3– Detection of PVY by nucleic acid hybridization by spotting of two fold dilutions of sap (1:4-1:1024) from different virus sources

Fig. 4– Detection of PVY by tissue printing with individual samples of *N. glutinosa* (petioles).

Fig. 5– Detection of PVY by spotting of purified PVY-RNA concentration

various strains of PVY[13] At nucleotide level, the percent sequence identity is in the same range for both 5′ UTR and the P1 coding regions of PVY[14] Therefore, we targeted the 5′ UTR and the PI protein of the PVY genome for PCR amplification and subsequent probe preparation Three combinations of primer pairs were used[15] a-d, amplifying PVY^N but not PVY^O, b-d, amplifying PVY^O but not PVY^N and c-d, amplifying all PVY strains (Y^O, Y^N and Y^C)

The RNA extracted from potato leaf samples was used for reverse transcription and subsequent PCR amplification Similarly, the RNA was also extracted from *N. glutinosa* leaves artificially inoculated with PVY^O In all the cases, b-d or c-d combinations resulted in the expected bands, suggesting the presence of the common strain of PVY (Fig 1) The primer combination c-d generated an amplified fragment of 969 bp The amplified product after cloning was labeled with α^{32} P-UTP and used as a probe Various field samples of potato were screened The same samples were also checked with the probe obtained from CIP, Lima (Peru) and the results were similar (Fig 2)

Leaf samples from different potato cultivars from Kufri (Fagu) farm were checked for the presence of PVY^O Out of a total 352 samples tested, 115 were found positive (Table 2)

Table 2– NASH testing of potato cultivars for the presence of PVY^o

Cultivar	Total samples	Virus positive	Virus free
Kufri Muthu	45	4	41
Kufri Ashoka	46	12	34
Kufri Lalima	26	26	-
Kufri Sherpa	51	-	51
Kufri Badshah	44	44	-
Kufri Pukhraj	31	29	2
Kufri Giriraj	45	-	45
Kufri Jyoti	64	-	64
Healthy control	5	-	5
Positive control	5	5	-

The efficiency of the probe was checked by taking sap in different dilutions from samples of *N glutinosa* and *Datura metel* artificially inoculated with PVY^{O} along with leaves from field collected potato plants, tubers and sprouts (Fig 3) The healthy extract did not produce any visible signal suggesting that the probe is highly specific in nature and there was no cross reaction with other common viruses The signal was also observed in direct tissue printing of infected samples (Fig 4), which is very important in seed farms etc where such lab facilities are lacking Samples could be directly spotted onto the membrane and sent to a lab where further processing for virus detection/confirmation may be done We could detect virus up to concentration of 60pg (Fig 5), showing the sensitivity of this technique

This technique showed great promise to test large number of samples with high specificity and sensitivity in a short period of time Besides ELISA, sensitive technique like NASH may be introduced in indexing program and samples also be rechecked through molecular hybridization

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Protein and free amino acids in lemmae of barley genotypes differing in grain protein content

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Abstract

Total protein and free amino acid contents were determined in lemmae at different stages of grain development in parent barley *(Hordeum vulgare* L) genotype NP-113 and its mutant genotype Notch-2 containing relatively lower grain protein content (6 6mg and5 3 mg per grain respectively) Whereas total protein content followed a somewhat zigzag pattern, total amino acid content increased up to 21 days after anthesis and decreased thereafter Total protein content was higher in NP-113 lemma compared with Notch-2 lemma On the other hand, free amino acid content was higher in Notch-2 lemma compared with NP-113 lemma It appears that lemma may play important role in determining protein content in cereal grains

(**Keywords** *Hordeum vulgare* L / barley/NP-113/Notch-2/lemma/protein/amino acids)

Introduction

In each floret of barley, the ovary and stamens are enclosed in two flowering glumes, the lower dorsal glume (lemma) and the upper ventral glume (palea) The lemma is nearly, fully developed at anthesis and contributes photosynthate to the carbon economy of the ear[1] A barley mutant genotype Notch-2 (5 3 mg protein grain^{-1}) and its parent genotype NP-113 barley (6 6 mg protein grain^{-1}) have been studied with respect to carbon and nitrogen metabolism in whole grain, endosperm, pericarp and leaves at different stages of grain development[2-6] However, such information is

lacking for lemma Present communication, reports total protein and free amino acid contents in these two barley genotypes (differing in grain protein content) at different stages of grain development Results indicate that nitrogen assimilation in lemma may play an important role in determining the protein content in grain

Materials and Methods

The NP-113 and Notch-2 barley (*Hordeum vulgare* L) plants were grown in earthern pots under identical conditions The ears were tagged on the day of anthesis, and lemmae were removed from tagged ears at 7, 14, 21, 28, 35 and 40 days after anthesis (DAA) Lemmae were collected only from middle part of the ear For determination of dry weight, samples of fifty lemmae were weighed and dried in a hot air oven at 80°C The dried samples were quickly transferred to a dessicator and allowed to cool at room temperature The dried samples were weighed again The process of heating, cooling and weighing was repeated until constant weight was obtained Total protein was calculated by multiplying total nitrogen values with a conversion factor of 5 837 Total nitrogen was estimated in dried samples by micro Kjeldahl method[8] Total free amino acids were estimated according to method of Yemm and Cocking[9]

Results and Discussion

Data on fresh weight, dry weight, total protein and total free amino acid contents are presented in Table 1 Fresh weight of lemma increased upto 21 DAA (except for a decrease in NP-113 between 7 DAA and 14 DAA), and decreased thereafter until maturity The fall in fresh weight during later part of grain development may be due to desiccation of lemma Marked difference in fresh weight of lemma, between the two genotypes was observed only at 7 DAA, when it was higher in NP-113 compared with Notch-2 Dry weight of lemma increased upto 35 DAA and decreased subsequently At 7 DAA, the dry weight of lemma was much higher in NP-I13 compared with Notch-2 However, it was reverse at 35 DAA The decrease in dry weight of lemma at 35 DAA may be due to incomplete separation of lower part of lemma, which remains fused to the base of the grain, during later stages of development

Total protein content of NP-113 lemma decreased up to 21 DAA, increased up to 28 DAA and decreased subsequently to a very low level at maturity. In Notch-2 lemma it followed a zigzag pattern Total protein content was higher in lemma of NP-113 compared with that of Notch-2 up to 28 DAA At this stage of grain development, NP-113 lemma recorded 76 % higher protein than Notch-2 The higher protein content

in lemma of NP-113 compared with Notch-2 suggests that higher protein content of lemma may contribute to higher protein content of attended grain (NP-113 grain has higher protein content than Notch-2 grain)

Table 1– Fresh weight, dry weight, total protein and total free amino acids in lemma of NP-113 and Notch-2 genotypes during grain development

Days after Anthesis	mg Lemma^{-1}							
	Fresh weight		Dry Weight		Total Protein		Total Free Amino Acids	
	NP-113	Notch-2	NP-113	Notch-2	NP-113	Notch-2	NP-113	Notch-2
7	30 76±1 05*	21 48±1 12	10 00±0 30	8 59±0 27	0 96±0 02	0 56±0 02	32 30±2 10	31 78±1 46
14	25 00±2 63	24 82±0 88	11 28±1 17	11 25±0 70	0 83±0 03	0 69±0 01	30 91±1 24	34 09±2 93
21	27 02±1 36	26 32±0 99	12 86±0 09	12 50±0 27	0 73±0 04	0 44±0 01	40 89±1 67	44 50±1 38
28	24 80±1 13	24 20±1 88	13 05±0 25	12 73±0 55	0 92±0 05	0 52±0 03	29 49±2 35	33 86±1 15
35	20 00±0 97	21 73±1 64	13 20±0 27	14 13±0 08	0 38±0 02	0 40±0 02	20 46±0 92	24 73±1 41
40	10 76±0 83	11 15±0 26	190 29±0 80	10 61±0 17	0 25±0 01	0 32±0 01	5 25±0 31	7 64±0 53

*Standard deviation for four replicates (Two samples of each genotype and two estimations of each sample)

Total free amino acid content of lemma increased up to 21 DAA and decreased thereafter until maturity (40 DAA) to very low levels in both genotypes except for an initial slight fall in NP-113 The low level of free amino acid content at maturity may be due to cessation of supply of assimilate from leaves to lemma, and a source-sink relationship may be operational with respect to these precursors of protein synthesis in leaves, lemma and attending grain There is high expression of genes for amino acid biosynthesis and photosynthesis indicating that the lemma/palea are major sources of nitrogen and carbon for the growing kernal[10] When changes in protein content and free amino acids are compared, it appears that upto 28 DAA, the lemma is not acting as consistent source of nitrogen to the kernal, rather it may be in a volatile situation acting as sink or source depending upon the nitrogen supply After 28 days it starts acting as a source of nitrogen to the kernal, which is evident from the senescence of lemma, as grain maturity approaches Proteolysis of chloroplast proteins begins in early phase of senescence and the liberated amino acids can be exported to the growing parts of the plant[11]. This could explain the zigzag pattern of protein content

observed, especially in Notch-2 lemma. Notch-2 lemma recorded appreciably high content of total free amino acids compared with NP-113 except at 7 DAA, when differences between the two genotypes were negligible. The higher content of free amino acids in Notch-2 lemma compared with NP-I13 may be the reflection of lower protein content in lemma of Notch-2 However, it is intriguing to find that despite higher content of free amino acids, it did not lead to higher protein content in Notch-2 grain. Therefore, markedly higher levels of free amino acid content[4] in endosperm and pericarp of Notch-2 compared with NP-I13, lead to the suggestion that lower protein content in Notch-2 compared with NP-113, may not be due to relatively lower transport of free amino acids from lemma to the grain, rather due to a block in the synthesis of protein in Notch-2 grain itself.

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Effect of fern plant extract on the growth and incidence of late blight fungus *Phytophthora infestans* on potato

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Abstract

Effect of extracts of three fern plants viz *Adiantum reniforme, Dryopteris filix mas* and *Polypodium decorum* on the growth and incidence of *Phytophthora infestans* were tested both in laboratory and field conditions The aquous extracts of all the test plants inhibited mycelial growth, spore germination and rate of infection of potato plant tissues Stimulatory effect of fern extracts on the growth of potato plants was revealed

(**Keywords** *Phytophthora infestans*/ potato plants/ferns/antifungal property)

Introduction

The higher plants contain a wide variety of chemical compounds These compounds are exuded by plants and show either stimulatory or inhibitory effects on other plants Schreiner and Sullivan[1] reported that plants produce certain chemicals which are toxic to other plants including microbes Since then a number of investigations have been made in this regard by several workers[2,3,4] The ferns are some such plants which are not only widely distributed but are also used by the local people as a medicinal plant for the treatment of certain diseases of man and cattle The present investigation aims at studying the effect of few fern plants commonly available in the crop fields of Assam, against *Phytophthora infestans,* the fungus causing late blight disease of potato

Materials and Methods

Preparation of plant extract- Extracts from leaf, stem and root tissues of three fern plants namely, *Adiantum reniforme, Dryopteris filix mas* and *Polypodium decorum* were prepared by the method followed by Parihar and Bohra[5] 5g of fresh plant parts were washed 2-3 times with tap water and then surface sterilized with 90% alcohol Subsequently the materials were grounded in 50ml distilled water and acetone

separately for aquous and acetone extracts respectively The acetone macerates were kept for 24h at room temperature to evaporate the acetone In the remaining residue 50ml of distilled water were added Macerates were squeezed through double layered muslin cloth and filtered through filter paper After filtration, aliquots were centrifused at 10,000rpm for 20min The supernatant were filtered through Whatman No 1 filter paper and then sterilized by passing through 0 2micron disposable filters The extracts(10%) thus obtained were used for the studies

In vitro studies - The antifungal activity of the plant extracts on the mycelial growth of *P infestans* was measured by adding 1ml extract to 10ml 'Limabean Agar media' (Savage *et al,*[6]) A small portion of sporulating colony was placed in the centre of the Petridishes containing the culture media Diameter of mycelial mat were measured after 72 hours

The rate of spore germination was evaluated by employing spore germination tests in cavity slides (Chandrol and Karkum[7]) Spores were collected by gently brushing the surface of infected leaf with a sterile needle and suspended in distilled water For each treatment, one drop of extract with loopful of spore suspension was put in cavity slides and covered with cover slip Suspensions prepared in a similar manner in sterile water served as control After incubation for 12 hours at 18°C, at least six microscope fields were examined to obtain germination percentage by using the following formula—

$$\textit{Percentage of spore germination} = \frac{\textit{Total number of germinated spore} \times 100}{\textit{No of spores/loop in spore suspension}}$$

In vivo studies—Seed tubers of healthy potato cultivar 'Up-to-date' were grown in earthen pots cotaining soil cowdung manure and compost at the ratio 2 1 1 Before sowing, each tuber was thoroughly sprayed with 50ml of 10% extract Successive sprayings were done at weekly intervals since the emergence of seedling for a period of 60 days Control was made with pots sprayed with water Late blight infection was measured by recording initial emergence of blight lesion on the plants Growth parameters of plants and incidence of late blight appearance were recorded after 75 days after sowing.

Results and Discussion

There was a significant reduction in mycelial growth of the fungus *P. infestans* when treated with aquous root and leaf extracts of *Adientum* and *Dryopteris*

Maximum reduction in growth was by leaf extracts of *Adiantum* (Table 1) In all the cases aquous extracts were far more effective than acetone extract Germination rate of sporangia of *P infestans* was reduced maximally by root extracts of *Dryopteris*

Table 1– Effect of plant extract on the mycelial growth(mm) and germination of sporangia(%) of *P infestans*

Plant extract		Control		Root		Stem		Leaf	
		A	B	A	B	A	B	A	B
		26 7	86 4	-	-	-	-	-	-
T_1	Aquous	-	-	09 5	68 3	22 5	76 8	06 5	54 7
	Acetone	-	-	18 3	57 8	16 4	81 5	20 6	70 0
T_2	Aquous	-	-	24 5	18 6	19 2	56 8	19 2	39 6
	Acetone	-	-	29 6	33 4	25 2	61 4	28 0	57 8
T_3	Aquous	-	-	08 6	73 3	21 4	67 6	11 6	64 5
	Acetone	-	-	21 4	82 5	25 7	75 2	27 3	67 0

C D at 5% T_1 = 0 07, T_2 = 0 03, T_3 = 0 11 A= Mycelial growth,

T_1= *Adiantum reniforme,* B= Germination of sporangia

T_2= *Dryopteris filix mas;*

T_3= *Polypodium decorum*

Field studies on the effect of plant extracts (aquous) on the rate of infection of potato plant tissues by *P infestans* showed that leaf extracts of *Adiantum* and *Dryopteris* significantly reduced blight infection in all the plant parts (Table 2) Reduction in the rate of infection however, was more evident in the leaf tissues of potato as compared to stems and tubers

The detrimental effects of higher plants of one species on the germination, growth or development of plants of another species have been an established fact The chemical compounds, released by the higher plants, also referred to as allelochemicals have been exploited in the form of extracts of different parts such as roots, stems, leaves, shoots etc in the stimulation and inhibition of growth characteristics such as seed germination, seedling growth, vigour etc (Mukherji & Sahai[8]) The present study on the growth parameters of potato plants sprayed with different fern extracts reveal both inhibitory as well as stimulatory effects Seedling emergence was accelerated in tubers treated with root and leaf extracts of *polypodium* but remained unchanged with

other extracts, rather it was delayed in tubers treated with leaf extracts of *Adiantum* and root and leaf extracts of *Dryopteris* A greater biomass and mean shoot length occurred in plants treated with *Polypodium* and *Dryopteris* stem and leaf extracts(Table 3) Vigorous leaf growth and more tubers per plant was observed in plants treated with all the extracts of *Dryopteris*

Table 2– Effect of plant extract on the rate of infection of potato plant tissue by *P infestans*

		Rate of infection(%) of plant tissue		
		Leaf	Stem	Tuber
Control		93 0	69 5	83 7
	T_1	27 3	56 4	65 8
F_1	T_2	49 4	61 2	59 6
	T_3	46 2	59 6	72 4
	T_1	42 3	47 3	61 4
F_2	T_2	31 0	45 4	69 3
	T_3	24 5	61 4	58 6
	T_I	87 4	74 5	89 3
F_3	T_2	91 2	67 6	67 5
	T_3	90 3	71 2	75 8

C D 5% T_1= 0 13, T_2= 0 07, T_3= 0 04

F_1 = *Adiantum reniforme* F_2= *Dryopteris filix mas* F_3= *Polypodium decorum*

T_1= *Root extract* T_2= *Stem extract* T_3= *Leaf extract*

The antifungal as well as stimulatory or inhibitory effect of a number of higher plants against crop plants and pathogens have been reported by a large number of workers The antifungal property of these plants is due to the presence of some phytochemicals or alkaloids that inhibit growth of plant pathogens Bohra and Purohit[9] obtained inhibition of growth of *Aspergillus flavus* by using extracts of *Azadirachta indica* The medicinal values of ferns are known to man from ancient times and extracts are used to cure certain diseases of man and cattles (Rice[10]) The antifungal property of *Adiantum lunatum, Marsilea minuta* and *Equisetum ramosissimum* in controlling human fungal pathogen *Candida albicans* have been reported by Parihar & Bohra[11] The present study reveals the antifungal property of

Adientum reniforme, Dryopteris filix mas and Polypodium decorum, few commonly growing ferns in the crop fields against *P infestans*

Table 3– Growth parameters of potato plants treated with different fern extracts

	Treatment	Seedling Emergence(%) (after 7 days)	No of leaves/plant	Leaf Size(cm)		Dry Biomass (g)	Mean Shoot Length(cm)	No of Tubers/ plant
				length	width			
	Control	76 7	42 5	6 8	3 4	2 65	25 3	14.2
	T_1	71 3	35 4	6 2	3 4	2 32	21 6	14 2
F_1	T_2	69 4	38 6	5 1	4 2	1 98	24 5	16 7
	T_3	56 5	28 9	6 9	3 0	2 65	26 1	16 5
	T_1	68 4	41 7	7 6	4 2	3 01	28 3	23 4
F_2	T_2	74 2	46 3	7 8	3 8	3 72	29 4	19.6
	T_3	52 0	47 6	6 9	4 7	4 01	31 2	26 1
	T_1	86 5	37 0	5 7	3 6	2 98	25 6	12 6
F_3	T_2	62 7	32 6	6 9	4 2	4 12	32 6	22 4
	T_3	91 2	40 5	7 1	4 0	3 86	29 7	19 0

C D 5% Treatment = 0 21 Data based on av of 25 plants for each treatment

F_1= *Adientum reniforme*, F_2= *Dryopteris filix mas*, F_3= *Polypodium decorum*

T_1= Root extract, T_2= Stem extract, T_3= Leaf extract

Management of diseases occurring in economically important plants and cultivated crops has been a challenging job for the scientists during past few decades The indiscriminate use of chemicals in the form of fungicides, pesticides, insecticides, and herbicides are causing serious problems in the overall environment Hence' alternative approaches, free from such problems, needs to be evaluated Use of crop allelopathy and allelochemicals for disease control may be a sound alternative, provided adequate and relevant informations are gathered

Phytophthora infestans is the causal agent of late blight which is by far the most serious disease of potato A lot of experimental works are in progress all over India to find suitable measures of controlling the disease The use of plant extracts, besides being harmless, the cost will be negligible in comparison to chemical fungicides

Moreover, it causes minimum distortion to the plant organs and also have virtually no side effects The present study suggests that species of fern plants such as *Adiantum, Dryopteris, Polypodium* etc , which are widely distributed anywhere, may be used to minimize the spread of the disease

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Phytoplankton primary production in relation to limno-chemical features in the context of fish yield potential of Hemavathy reservoir, Karnataka

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Abstract

Studies on phytoplankton primary production in Hemavathy reservoir during 2000-02 revealed that both the gross and net primary productions were more (677 09 & 424 63 $gC/m^2/y$) in the first year as compared to second (571 80 & 355 01 $gC/m^2/y$) year primarily because of favourable climatic and edaphic conditions coupled with more inflow and less outflow in the first year Community respiration was not varied widely (253 04 and 216 81 $gC/m^2/y$) in the two years respectively Primary production showed high correlation with specific conductance, total alkalinity, and hardness Potential fish yield was 100 kg/ha/y against the present harvest at 20 kg/ha/y leaving enough scope of augmenting fish production through sustainable management practices

(**Keywords** primary production/ limno-chemistry/ fish yield potential/reservoir),

Introduction

The peninsular State Karnataka is adorned with its impressive reservoir resources having tremendous untapped potentiality Phytoplankton primary production coupled with systematic study on limno-chemistry could be utilized as indices of trophic status including assessment of fish production potential of such water bodies Well documentation in this aspect has been made in some tropical Indian reservoirs[1-5] Due to location specificity with wide variations in their morphometry, catchment characteristics, degree of climatic and anthropogenic interventions these water bodies are to be studied individually to unravel the problems related to fishing management Barring the works on Tungabhadra[6], Vanivilas Sagar[7], Markonahalli[8], Supa[9] and Manchanbele[10] scientific information in this aspect on Karnataka reservoirs is very meagre This investigation on primary production was made on 48 occasions in even months in Hemavathy reservoir, Hassan, Karnataka emphasizing nature of productivity profiles in relation to limno-chemical features Fish production efficiency

of this reservoir was also worked out to formulate sound and sustainable management norms for development of this water body

Study area

Hemavathy reservoir (latitude 12° 40' N and longitude 76° 30' E) across the rivers Hemavathy and Yagachi, the tributaries of river Cauvery was constructed in 1979 at Gorur, 20 km from Hassan district, Karnataka Though, basically meant for irrigation purpose, a hydel project (4 0 MW) has come up in 1997 on the left bank of the reservoir Of late, construction of another hydel project is at full swing with 4 0 MW capacity at the right bank canal mouth Certain salient morphometric and hydrographic characteristics of this reservoir has been presented in Table 1

Table 1– Salient morphometric and hydrographic features of Hemavathy reservoir

Latitude	12° 40' (N)
Elevation (m asl)	890 62
River bed (m asl)	850 42
River	Hemavathy and Yagachi
Year of construction	1979
Catchment (C) (km^2)	2800
Area (A) (ha)	8502
C/A	33
Maxium depth (m)	40 20
Mean depth (m)	12 40
Capacity (10^6 m^3)	1050 63
Total inflow (10^6 m^3)	3646
Flushing rate	3 47
Volume development	0 92
Shore development	1 60

The lentic of Hemavathy is located in a sprawled area whereas, its lotic is confined in mostly gorgy basin. In lentic zone, the shoreline is even with gentle slope having extended littoral sectors The catchment to reservoir ratio (C/A), an index of allochthonous inputs is moderate (33)

The climatic condition surrounding the reservoir is mainly dry The annual rainfall in the catchment varies from 200 to 450 cm, decreasing from west to east Major catchment area is hilly with forest cover along with coffee plantations and cultivated land Deep to very deep soil predominates in this area Soil texture is mostly clay-loam to sandy-clay-loam

Materials and Methods

The experiment involved monthly *in situ* measurement of primary production for two consecutive years, from August 2000 to July 2001 (first year) and August 2001 to July 2002 (second year) following Vollenweider[11] using light and dark bottles Two sites were selected in the lentic zone, one in the Hemavathy river side (Lentic Right, LR) and the other one in the Yagachi river side (Lentic Left, LL) (Fig 1) The bottles were incubated for 4 h between 10 00 and 14 00 hours Dissolved oxygen was estimated immediately after exposure by Winkeler's method Average hourly rates were multiplied by the hours of sunshine to get the daily rates Estimation of per unit surface area for primary production as well as community respiration was made by integrating the values obtained at different depths of the water column starting from immediate subsurface followed by 1 m, 2 m, 3 m and 4 m below from the surface Annual measurements were calculated by summing up the monthly values following Peterson[12] Physico-chemical features of water were determined as per standard methods[13]

Results and Discussion

Environmental character

The reservoir started filling by the end of June or early July due to early rains in Hassan, reaching its maximum water level of 40 20 m by the end of August and early September Water level was not stabilized in subsequent post-monsoon months due to heavy draw down by hydel project as well as through irrigation canal

The reservoir received lesser inflow (1609 08 x 10^6 m^3) in the second year as compared to favourable inflow (2388 27 x 10^6 m^3) in the first year when water level touched the FRL (Fig 2) Water temperature at the surface varied sinusoidally ranging between 22 0°C and 28 3°C with summer high and monsoon/winter low Transparency did not show any clear seasonality due to erratic rainfall in the second year and gravelly soil character in the catchment (Fig 3) Secchi disc depth recorded minimum at 0 95 m and maximum at 2 55 m with an overall mean at 1 94 m Thus the euphotic zone extended between 3 0 and 6 0 m Moderately rich electrolyte (mean specific conductance 96 60 µS/cm) with poor total alkalinity (mean 36 88 mg/l) as well as hardness (mean 27 67 mg/l) was the characteristics of this reservoir, not significantly differed from other Cauvery Basin (CB) reservoirs of Karnataka[14] The nutrient regime was moderate with the enrichment observed during monsoon as well as in some summer months signifying moderate productivity of this reservoir (Table 2)

Table 2– Salient limno-chemical features of water of Hemavathy reservoir (2000-02)

Parameters	Range	Average
Temperature (°C)	22 00-28 25	24 98
Transparency (m)	0 95-2 55	1 94
pH	6 75-8 47	7 58
Specific conductance (µS/cm)	65 00-130 00	96 60
Dissolved oxygen (mg/1)	6 45- 7 85	7 17
Total alkalinity (mg/l)	28 00-50 00	36 88
Total hardness (mg/1)	16 00-38 00	27 67
Nitrate-N (µg/1)	27 50-350 00	159 29
Phosphate-P (µg/1)	10 00-200 00	64 58
Total-P (µg/1)	10.00-590 00	132 40
Silicate-Si (mg/1)	0 35-6 31	2 62

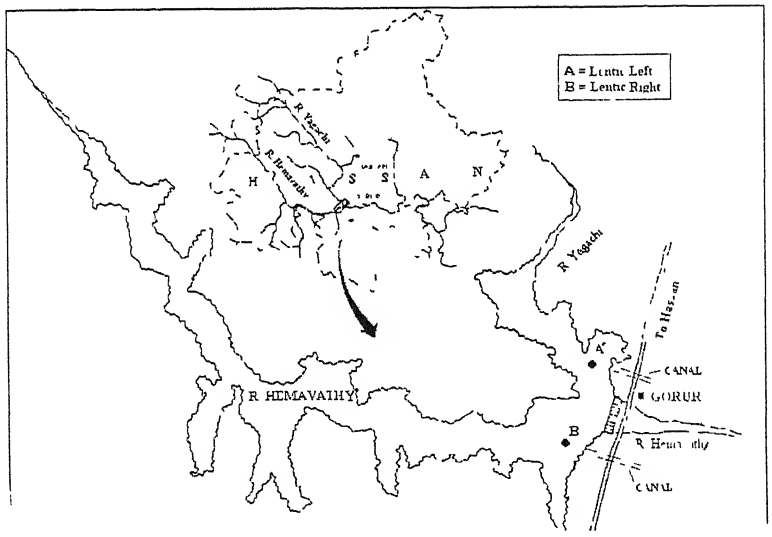

Fig 1– Hemavathy reservoir

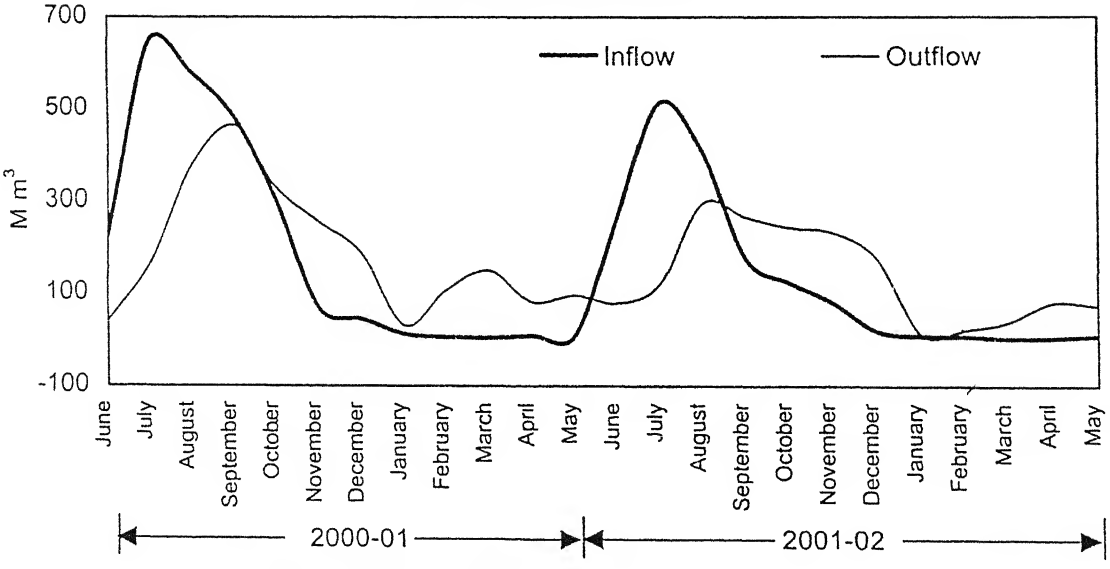

Fig 2– Inflow and outflow in Hemavathy reservoir (2000-02)

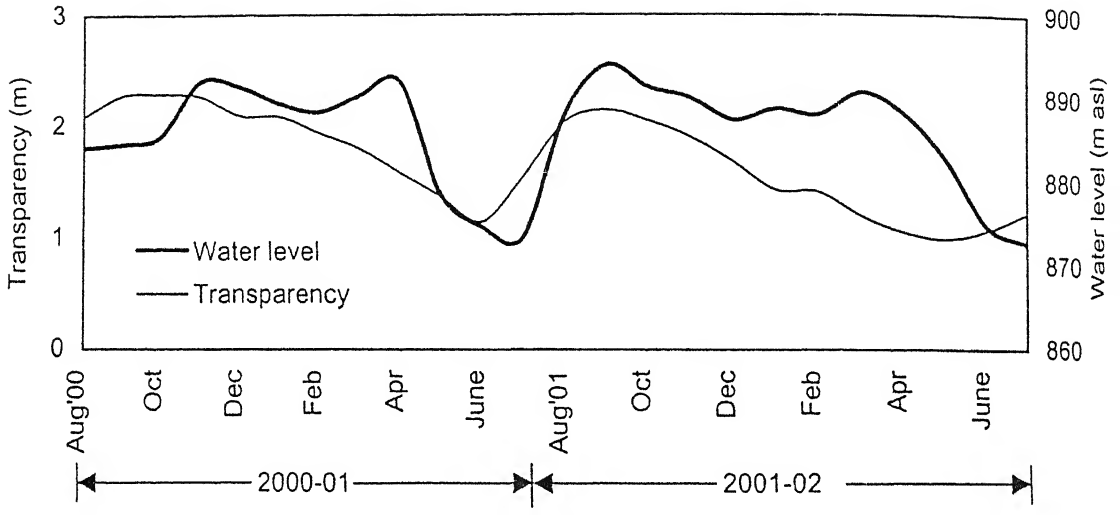

Fig 3– Temporal variation in water level & transparency in Hemavathy

Primary production

Gross primary production (GPP)

Vertical profile of gross primary production showed marked monthly fluctuation in both the sectors with single or bimodal peak Single peak was observed in 2000-01 during September (LR), December, January, March and June (LL), single but extended peak was noticed in February, November, May and July (LL) On the contrary, dichotomous profiles with more than one maxima were common in August, September (LL), October, January April, May, June and July (LR) With some modifications, the vertical depth profiles in 2001-02 with single peak was noticed only in February (LR), single but extended peak was more prominent in September (LL), November, December, January (LR) and February (LL) Dichotomous profiles with more than one maxima were found in August, September, (LR), October, December, January and March (LL) (Fig 4)

Bimodal productivity profiles are explained in terms of change in light regimen and the eco-physiography of the phytoplankton Production curves with multiple maxima are said to be common in small lakes[15] According to Finnegg[16], dichotomous curves are characteristics of certain lakes and it is also equally clear that, in a lake, the shape of production curve can change seasonally. In the present study, the thermal stratification was less marked due to low variation in surface and bottom temperature of water coupled with continuous drawing of reservoir water for hydel purpose, thereby allowing the even distribution of bimodal peaks year round in this water body

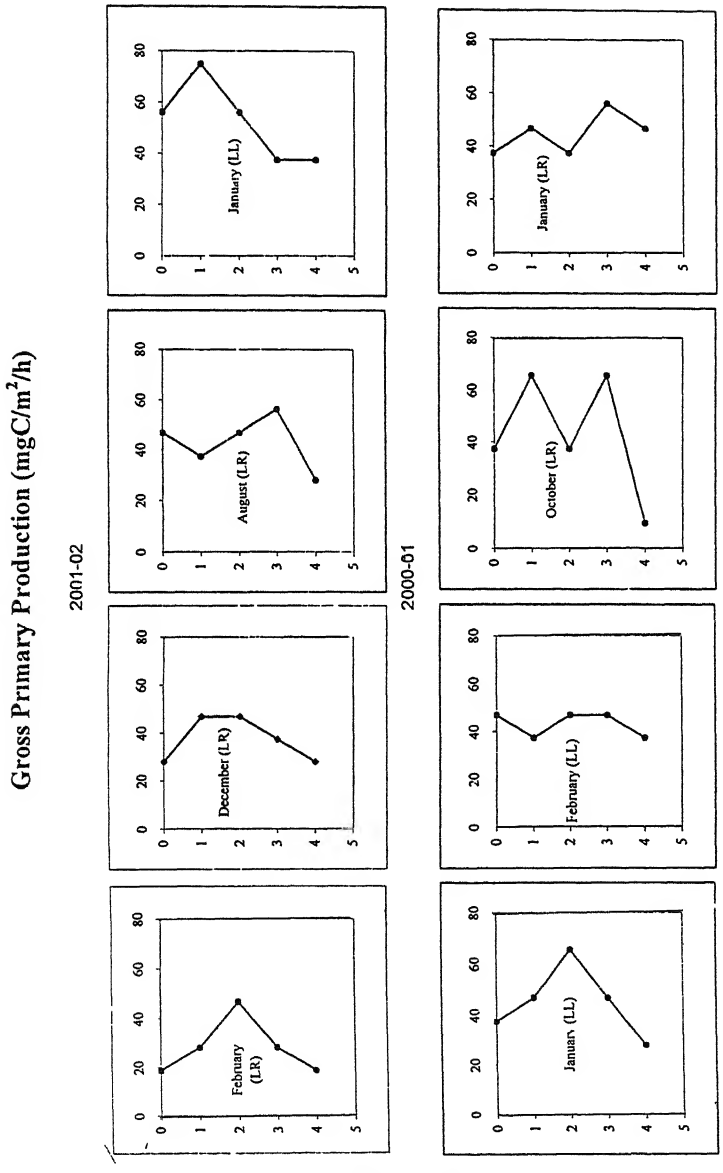

Fig 4– Some selected production profiles with depth in Hemavathy reservoir

Sub surface maxima occurred in most cases (42 out of 48) at 1 0 & 3 0 m depth and 2 0 & 3 0 m depth from the surface in the first and second year respectively suggesting evidence of surface photo inhibition as also observed by Lewis[17] in lake Lanao, Philippines In a Sri Lankan reservoir, Dokulil *et al*[18] observed photo inhibition in 10 out of 22 experiments Same was also true in case of Manchanbele reservoir (MBR), a CB reservoir, near Bangalore, Karnataka where sub-surface maxima were encountered in 17 out of 26 experiments[10] Some of the depth profiles were characterised by lower production per unit area and some were with extended area having substantial production per unit area Thus, hourly integrated gross production (ΣA)(mgC/m^2/h) exhibited wide variations from August to July to the tune of 150 & 300 mg (mean 231 88) and 165 & 292 5 mg (mean 195 83) in the first and second year respectively The daily integral production ($\Sigma\Sigma$ A) (mgC/m^2/d) fluctuated between 1200 & 2400 and 1320 & 2340 mg in the first and second year respectively with an annual mean of 1855 & 1567 mg in those two years respectively

Thus, there was change in the shape of the vertical profiles encountered during first and second year In the first year, the profiles were characterised by higher production per unit area in contrast to lower production per unit area in the second year with the absence of marked maxima and truncated distribution (as depicted from Fig 5a) when the reservoir received poor inflow

Gross primary production exhibited distinct seasonality with increasing trend during post-monsoon months (October-January) in both the years and troughs in monsoon (August-September) and early pre-monsoon (April-May) periods Temporal changes of primary production in tropical lakes as designed by Melack[19] are i) well defined seasonal fluctuations caused by rains or vertical mixing, ii) narrow seasonality and iii) abrupt changes with narrow seasonality Though, Indian reservoirs exhibiting all the three patterns of production[2], Hemavathy reservoir showed the first type where low photosynthetic rate[20] was observed in monsoon as influenced by rain with the dilution of nutrients and in early pre-monsoon with low phytoplankton density[21] due to low nutrient content.

Gross primary production showed a little difference in peaks and troughs so far as spatial variation is concerned as observed in lentic right and lentic left sectors (Fig. 5a) A sharp peak in June in both the years as well as in both the sectors was due to lowest water level corresponding to high plankton density[21]

The temporal and spatial variation in Amax, the light saturated rate of photosynthesis, which is an indicator of the capacity of a lake to produce and sustain algae[22] has been depicted in Fig 5b. In the 1st year, Amax maintained markedly and

consistently higher rate than the second year, probably due to higher algal biomass in the first year The maximum values of Amax ($mgC/m^3/h$) were recorded in June in first year were 112 50 and 103 13 in lentic left and lentic right respectively coinciding with the highest plankton density[21] in this month In the second year, Amax was maximum in June in the lentic right sector (93 75) and January in the lentic left sector (75 00) In MBR, the maximum Amax value recorded was 230 $mgC/m^3/h$ by Krishna Rao *et al* [10] A maximum value of Amax (989 $mgC/m^3/h$) has also been recorded[23] in a shallow, small Nelligudda reservoir, near Bangalore

Net primary production (NPP)

The hourly net primary production ($mgC/m^2/h$) varied widely from 62 5 to 222 5 and 90 to 160 in the first and second year respectively and was distributed over a much narrower depth than gross production The average annual net production was 145 42 and 121 58 $mgC/m^2/h$ in the first and second year respectively (Fig 5c)

Community respiration (CR)

The rates of respiration in the euphotic zone were in higher side during August, September, January (LR), March (LR), April and May (LL) in the first year and August, January, April and June (LR) in second year The average CR was higher in the first year (86 66 $mgC/m^2/h$) as compared to second year (74 25 $mgC/m^2/h$) (Fig 5c) The community respiration rate in Hemavathy reservoir signifies the dominance of autotrophic food chain rather than heterotrophic one[21]

P-R ratio

Odum[24] designed the idea of determining P-R ratio in order to define a community type and also the concept of autotrophy and heterotrophy The P-R ratio fluctuated widely to the tune of 1 6 to 4 2 in 2000-01 than the second year 2 0 to 3 3 The ratio varied greatly with seasons, higher values in post-monsoon and late summer (Fig 5d) The trough in monsoon was due to dilution effect of nutrients getting reflected in the low phytoplankton density Same was true in early pre-monsoon also with low population of phytoplankton community[21] In productive waters P-R ratio remains low[25] In this study, the annual mean of this ratio was around 2 6, suggesting that the water body was moderately productive.

Annual primary production and energy transformation

Data on annual primary production (Table 3) computed from the average daily rates indicated that both GPP and NPP varied greatly in both the years. The values were 677 09 & 424 63 $gC/m^2/y$ in the first year and 571 80 & 355 01 $gC/m^2/y$ in the second year

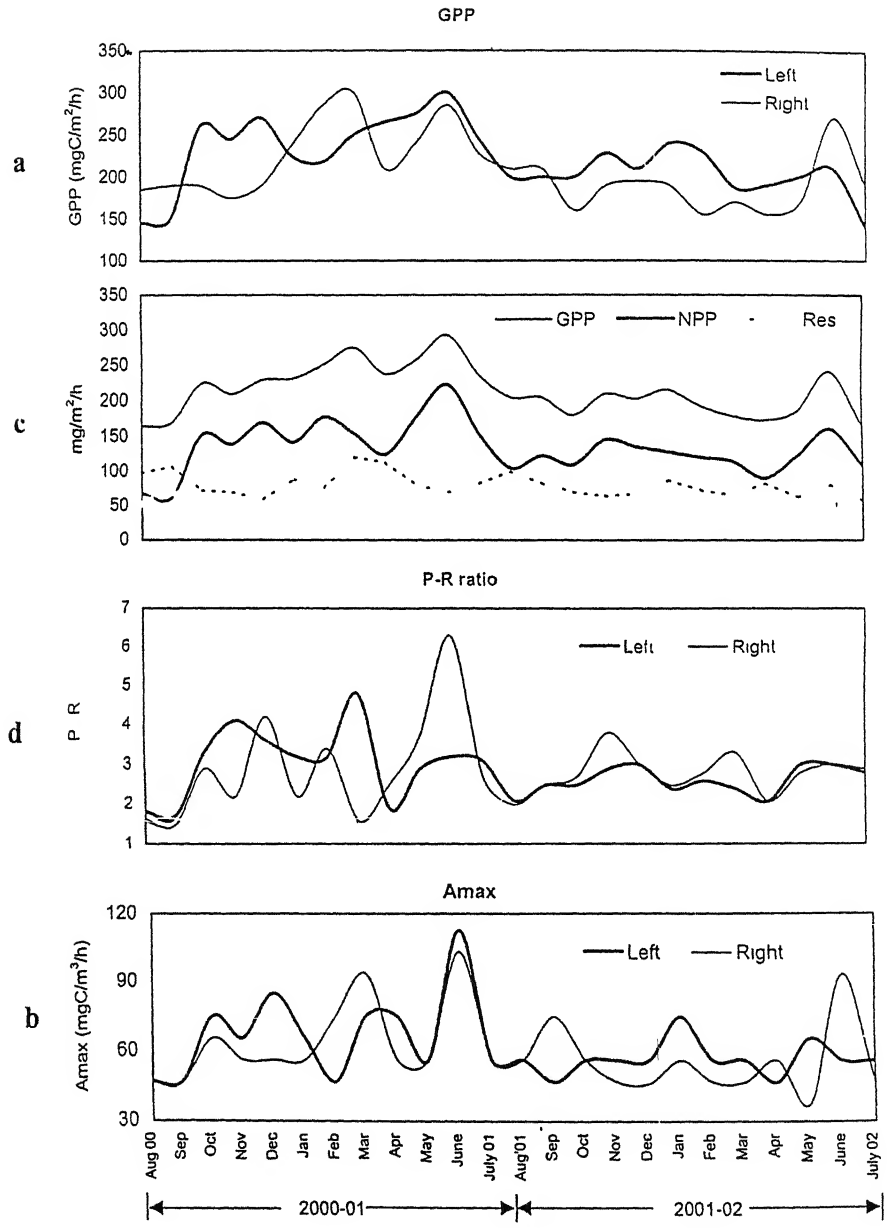

Fig 5– Temporal variations of GPP, NPP, P R and Amax in Hemavathy reservoir

Energy in the form of photosynthetic primary production was estimated to be 67 71 x 10^6 and 57 18 x 10^6 Kcal/ha/y in this reservoir with assimilation efficiencies 62 71% and 62 08% in 2000-01 and 2001-02 respectively

Table 3– Annual primary production and community respiration in Hemavathy reservoir

		GPP	NPP	CR
2000-01	$gC/m^2/y$	677 09	424 63	253 04
2001-02	$gC/m^2/y$	571 82	355 01	216 81

Correlation matrix

Primary production showed strong positive correlation with dissolved electrolytes ($r = 0\ 745$, $p<0\ 01$)and total alkalinity ($r = 0\ 656$, $p<0\ 01$) Same was true in case of net primary production and total hardness also Weak correlation observed amongst production and dissolved nutrients might be due to their quick turn over The importance of nitrogen and phosphorus as limiting factors for primary production is well documented[26] Absence of correlation between production and transparency noticed in Hemavathy has also been observed by Das[5] suggests that variation in transparency was not due to algal biomass alone (Table 4)

Fish production potential in Hemavathy

The very basis of reservoir management for augmenting fish production sustainably is to predict fish yield potential Several indices have been proposed time to time to correlate fish yield to physical, chemical as well as biological features[27] However, each model has its own limitation in prediction in a particular situation[28-29] Mc Connel *et al* [30] found the conversion efficiency from GPP to fish yield in the range between 0 1 and 1 6% in natural waters At present, in Hemavathy, with provisional management, the maximum fish harvest was 20 kg/ha/y amounting a total of 100 t of fish catch annually

The yield potential estimated through GPP provides a fairly dependable limnological guide to fish productivity in Indian reservoirs[31] The targeted potential fish yield (TPFY) estimated through Melack's[19] model, MDI[32] and biomass calculations[33] were 36, 25 and 140 kg/ha/y respectively Assuming a judicial conversion efficiency of 0 2% GPP, the TPFY of Hemavathy would be around 100 kg/ha Fish conversion efficiency from GPP based on present landings of fish would have given the real conversion efficiency of the target reservoir At present 20% of TPFY is being harvested from Hemavathy So, there is still enough scope of

increasing fish production even up to 50-60% of TPFY, which could be achieved easily through scientific management norms

Table 4– Correlation matrix

	GPP	NPP	CR	P R
Temperature	-0 292	-0 375	0 193	-0 372
Transparency	-0 164	-0 246	0 183	-0 197
pH	0 059	0 165	-0 224	0 161
Sp conductance	0 745**	0 663**	0 145	0 542**
Diss oxygen	-0 073	-0 151	0 165	-0 257
Total alkalinity	0 656**	0 576**	0 144	0 526**
Total hardness	0 264	0 409*	-0 318	0 514*
Ca^{++}	0 394	0 447*	-0 125	0 525**
Mg^{++}	-0 017	0 160	-0 377	0 241
Cl^{-}	-0 132	-0 037	*-0 202*	0 051
Nitrate-N	0 057	0 029	0 052	*-0 004*
Phosphate-P	-0 283	-0 432*	0 322	-0 526**
Total-P	*-0 106*	-0 114	0 016	-0 192
Silicate-Si	-0 521**	-0 312	-0 421*	-0 149

Significant at (*) 0 05 = 0 4043 and (**) 0 01 = 0 5151

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Effect of amino acids on growth and siderophore production of fungi

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Abstract

Five fungi, *(Aspergillus* sp ABp4, *Penicillium oxalicum, Aureobasidium pullulans, Mycotypha africana* and *Syncephalastrum racemosum)* were undertaken for examining the effect of 8 amino acids (alanine, arginine, glycine, histidine, leucine, ornithine, serine and valine) on growth and siderophore production The amino acids increased both growth and siderophore production in 18 fungi, decreased both in 7, increased siderophore with decreased growth in 8 and vice versa in 7 fungi However, alanine always showed a positive effect on siderophore production Total suppression of siderophore production was noted for 3 fungi *A pullulans* (by glycine and valine), *M africana* (arginine, glycine, leucine and serine) and *S racemosum* (by glycine and valine).

(**Keywords** fungi/ growth/ siderophores/ amino acids)

Introduction

Siderophores are low molecular weight (<1000D) virtually Fe (III) specific ligands produced by microorganisms to combat low iron stress[1,2] They facilitate the solubilization and transport of iron into the cell by a cognate transport system No system analogous to siderophores has been known for any metal ion, thus, making iron unique in requiring such specific ligands

Organic acids, sugars, enzymes and vitamins serve as growth substrates and are responsible for enhancing microbial activity in the soil or in the rhizosphere[3] Bacteria and fungi predominate in the soil because they can utilize these organic compounds Root exudates affect growth and metabolite production of these organisms and hence determine the size of microbial populations in the bulk soil or in the rhizosphere[4] Siderophore production is influenced by minerals[5], amino acids[6], peptones[7] and carbon sources[8,9] The biosynthetic pathways of siderophores are tightly connected to aerobic metabolism involving molecular oxygen activated by mono-, di- and N-oxygenases and the use of acids originating from the final oxidation of the citric acid

cycle, such as citrate, succinate and acetate Moreover, all siderophore peptides are synthesized by non-ribosomal peptide synthetases and in the case of fungal siderophores are mainly built up from ornithine, a non-proteinogenic amino acid[10] Thus, amino acids play a key role in simple bidentate ligands as building blocks

The present paper deals with the influence of amino acids on siderophore production by test fungi

Materials and Methods

Fungi Five fungi, (*Aspergillus* sp ABp4, *Penicillium oxalicum, Aureobasidium pullulans, Mycotypha africana* and *Syncephalastrum racemosum*) were undertaken for the study All these fungi were grown and maintained on potato dextrose agar medium[11] and stored at 4°C These were potent siderophore producers as confirmed by the $FeCl_3$ test[12], CAS assay[13] and CAS agar test[13,14]

Media for siderophore production The first three fungi, belonging to *Asco-* and *Basidiomycota*, were grown in Grimm-Allen medium[15] containing (per liter of distilled water) K_2SO_4 1g, ammonium acetate 3g, K_2HPO_4 3g, citric acid 1g, sucrose 20g and adjusted to pH 6 8 with ammonia The medium was then supplemented with thiamine 2mg, $CuSO_4$, $5H_20$ 0 005mg, $MnSO_4$, $4H_20$ 0.035mg, $ZnSO_4$, $7H_20$ 2mg, $MgSO_4$, $7H_2O$ 80mg The remaining two belonging to *Zygomycota*, were grown in Modified M9 medium[16] containing (per liter of distilled water) glucose 10g, Na_2HPO_47g, KH_2PO_43g, NaCl 0 5g, NH_4Cl 1g, $MgSO_4$ 0 25g, $CaCl_2$ 0 015g, and adjusted to pH 7 2 The medium was supplemented with thiamine 0 005g and $ZnCl_2$ 0 015g

All glass wares were soaked overnight in 6M HCI and rinsed with distilled water several times to remove traces of iron[13]

The above media were decontaminated of iron by adding 8-hydroxyquinoline dissolved in chloroform When shaken this formed ferrous or ferric hydroxyquinates After separation, the chloroform layer was removed and the medium was washed repeatedly with chloroform to ensure complete removal of iron complexes and any residual 8-hydroxyquinoline, which could inhibit growth[17] All the above media were dispensed in 50 ml volume in 500 ml flasks

Both the media were supplemented with 1g/L of each amino acid (alanine, arginine, glycine, histidine, leucine, ornithine, serine and valine) individually After 15 days, the growth and siderophore production were noted The cultures were filtered using Whatman No 42 filter paper and the mycelial mats were dried at 60°C for 48h

for measuring dry weight[18], while the cell-free culture filtrates, obtained by filtration, were examined for the siderophores by the CAS assay The amount of siderophore was extrapolated by using a standard curve prepared for desferal (as hydroxamate) and rhizoferrin (as carboxylate) and denoted as µg/ml

Results and Discussion

The data (Table 1, Fig 1) suggest that (I) amino acids increased both growth and siderophore production, (II) inhibited both or (III) increased one and (IV) decreased the other Extreme effect was noted when siderophore production was curtailed in presence of certain amino acids

Among the first category, *Aspergillus* sp ABp4 was the only fungus that showed rise in both growth and siderophore production with all the eight amino acids The next best positive effects were seen with *A pullulans* and S *racemosum,* in which also both growth and siderophore production were increased, in presence of 5 and 4 amino acids, respectively, while *P oxalicum* showed similar increase in both with only one amino acid (valine) The opposite response i e decrease in both growth and siderophore production, was most prominent in *M africana* with four amino acids (arginine, glycine, leucine, serine), followed by *P oxalicum* with two (arginine, ornithine) and *S racemosum* in one case (valine)

Rise in siderophore production with fall in growth was recorded maximally with four amino acids (alanine, histidine, ornithine, valine) in *M africana,* followed by *P oxalicum* with three amino acids (alanine, leucine, serine) and *A pullulans* only with leucine

The increased growth but reduced siderophore production was noted with three amino acids (glycine, histidine, leucine) in case of *S racemosum,* and with two amino acids in *P oxalicum* and *A pullulans*

Maximum siderophore production was seen in *M africana* (4050 µg/ml) in presence of histidine among zygomycetes, whereas it was found to be maximum in *P oxalicum* (191.24 µg/ml) in presence of leucine among the asco- and basidiomycetes

Maximum growth was observed in presence of leucine (516 4 mg/ 50 ml) in *Aspergillus* sp. ABp4 followed by *A pullulans* (491 9 mg/50 ml) in presence of glycine

Table 1– Effect of amino acids on growth (mg/50ml) and siderophore production (µg/ml)

Amino Acid→ / Test Fungi ↓		Control	Alanine		Arginine		Glycine		Histidine		Leucine		Ornithine		Serine		Valine	
Aspergillus sp ABp[4]	Sid	15 55	22 36 43 8%	↑	65 85 323 5%	↑	63 18 306 2%	↑	32 32 107 8%	↑	60 51 289 1%	↑	39 85 156 2%	↑	35 96 131 2%		78 25 403 2%	↑
	Growth	45	435 866 6%	↑	479 9 966 4%	↑	447 4 894 2%	↑	477 0 960%	↑	516 4 1047 5%	↑	491 8 992 8%	↑	482 8 972 8%		484 8 977 3%	↑
Penicillium Oxalicum	Sid	78 25	107 6 37 5%	↑	37 91 51 55%	↓	65 37 26 3%	↓	54 68 30 12%	↓	191 24 144 2%	↑	64 15 52 5%	↓	120 8 54 4	↑	103 3 32 1%	↑
	Growth	315	305 2 3 11%	↓	176 5 43 97%	↓	398 1 26 3%	↑	339 8 7 8%	↑	304 5 3 33%	↓	241 2 23 43%	↓	267 8 14 98%	↓	368 2 16 8%	↑
Aureobasidium pullulans	Sid	24 1	61 0 153 1%	↑	32 8 36 1%	↑	-		48 1 99%	↑	63 7 164 1%	↑	43 7 81 3%	↑	43 3 79 6%	↑	-	
	Growth	376 4	439 3 16 7%	↑	415 1 10 3%	↑	491 9 30 7%	↑	513 8 36 5%	↑	312 2 17 1%	↓	442 8 17 6%	↑	475 7 26 4%	↑	467 2 24 1%	↑
Mycotypha africana	Sid	386 3	1113 8 188 4%	↑	-		-		4050 948 5%	↑	-		3513 7 809 7%	↑	-		1766 3 357 3%	↑
	Growth	397 6	176 3 55 7%	↓	202 9 48 9%	↓	342 6 13 8%	↓	355 9 10 5%	↓	141 9 64 3%	↓	328 0 17 5%	↓	211 6 46 8%	↓	240 1 39 6%	↓
Syncephalastrum racemosum	Sid	438 8	1698 8 287 1%	↑	1173 8 167 5%	↑	-		352 5 19 7%	↓	356 3 18 8%	↓	393 795 6%	↓	476 3 8 6%	↑	-	
	Growth	170 1	181 1 6 5%	↑	213 7 25 6%	↑	202 0 18 8%	↑	191 6 12 6%	↑	233 1 37%	↑	198 5 16 7%	↑	188 6 10 9	↑	166 9 1 8%	↓

Fig 1– Effect of amino acids on mycelial growth (dry wt, mg/50 ml) and siderophore production at 630 nm (μg/ml)

↑ Growth, Siderophore 8/40 ↓ *M africana* (4) ala, his, orn, val *P oxalicum* (3) ala, leu, ser *A pullulans* (1) leu	Growth, Siderophore 7/40 ↓ ↓ *M africana* (4) arg, gly, leu, ser *P oxalicum* (2) arg, orn *S racemosum* (1) val
↑ ↑ Growth, Siderophore 18/40 *Aspergillus* sp ABp4 (all test amino acids) *A pullulans* (5) ala, arg, his, orn, ser *S racemosum* (4) ala, arg, orn, ser *P oxalicum* (1) val	↑ Growth, Siderophore 7/40 ↓ S *racemosum* (3) gly, his, leu *P oxalicum* (2) gly, his *A pullulans* (2) gly, val

If we see the performance of the various amino acids (supportive/inhibitory), it was not uniform Only alanine always had a positive effect on siderophore production Others decreased or increased the siderophore production for the different fungi

Total suppression of siderophore production by amino acids was noted for *A pullulans* (by glycine and valine), *M africana* (arginine, glycine, leucine and serine), and *S racemosum* (glycine, valine)

Dave[19] noted invariably higher growth and siderophore production for four fungi viz *Aspergillus ochraceous, Penicillium citrinum, Penicillium camembertii* and *Neurospora crassa* However, Korat[20] observed that the influence of the amino acids was not always stimulatory As we have noted, she also found increase in growth as well as siderophore production, enhancement of one, but reduction in the other (growth/ siderophore production) or decrease in both growth and siderophore production She also observed that alanine always had a positive effect i e enhanced both growth and siderophore production for the three test fungi viz *Mucor mucedo, Rhizopus oryzae* and *Syncephalastrum* sp We too have made a similar observation with alanine, which enhanced siderophore production for all the 5 test fungi

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Effect of application of L-phenylalanine and cinnamic acid on phenylalanine ammonia lyase activity in pea *(Pisum sativum)* and conidial germination of *Erysiphe pisi*

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Abstract

Foliar application of L-phenylalanine and cinnamic acid increased phenylalanine ammonia lyase (PAL) activity in pea leaves and reduction of conidial germination of the pea pathogen *Erysiphe pisi* was correlated with increased PAL activity in pea leaves

(**Keywords** Cinnamic acid/L-phenylalanine/phenylalanine ammonia lyase/*Erysiphe pisi*)

Introduction

Plants possess a wide array of defence against diseases by a combination of constitutive and inducible mechanisms Among important products of these defence responses are enzymes that are involved in the flow of carbon from primary to secondary metabolism in plants and secondary metabolites such as phenolics[1] These compounds play an important role in plants against fungal infection[2] Phenylalanine ammonia lyase (PAL, EC 4 3 1 5) is the first committed enzyme in the phenylpropanoid pathway leading to the conversion of L-phenylalanine into t-cinnamic acid with the elimination of ammonia[3] Many plant-specific phenylpropanoid branch pathways and their corresponding functional diversity are basically originated from the core phenylpropanoid metabolism initiated by PAL[1] In both constitutive as well as systemically induced defence responses, magnitude of accumulation of phenolics may be governed by the PAL activity and essentially depend upon the availability of the precursor (L-phenylalanine)[2,3] The end-product

cinnamic acid and its derivatives, e g , cinnamic, coumaric, vanillic and synapic acids and other abiotic factors also affect PAL activity[4,5] and therefore, play important role in defense mechanisms Present investigations conducted to determine the effect of both, the precursor and the end-product cinnamic acid, on PAL activity in pea *(Pisum sativum)* The response of exogenous applications of L-phenylalanine and cinnamic acid on germination of conidia of *E pisi* was also assessed

Materials and Methods

Pea (*P sativum*) (var Arkel) seeds were sown in plastic pots (15 cm dia) and kept at 25 ± 2°C in a glasshouse in a light source of Philips Son- T lamps on a 12 h light/dark photoperiod Twenty one day old plants were sprayed with different concentrations (50,100 and 150 ppm) of L-phenylalanine and cinnamic acid prepared in 0 1% Tween-20 Fresh leaves of pea were harvested after 24, 48, 72 and 96 h of treatment for the analysis of phenylalanine ammonia lyase activity by using spectrophotometer The enzyme extract was prepared as per the method of Havir *et at* The enzyme was extracted by suspending 0 5 g of fresh pea leaf tissues in borate buffer (0 2M, pH 8 7, 4ml) at 4°C Reaction mixture containing 1ml enzyme extract, 2 0 ml borate buffer, and 0 2 ml 0 1M L-phenylalanine was incubated at 32 ± 1°C for 30 min After incubation, the enzyme reaction was stopped by adding 0 5 ml of 1M Trichloroacetic acid The cinnamic acid thus formed at the end of incubation time in the whole reaction mixture was fractionated thrice with ethyl acetate (5 ml x 3) The upper organic layer was pooled together and evaporated to dryness under vacuum The ethyl acetate fraction was further dissolved in 1 0 ml methanol and volume of each sample was made up to 2 0 ml in methanol prior to spectroscopic analysis at 290 nm

Different sets of healthy plants (21 day old) were sprayed with different concentrations of L-phenylalanine and cinnamic acid Conidia of *E pisi* were gently tapped from a heavily infected pea plant holding over the leaves of healthy pea plants 24 h after the spray of chemicals The concentration of *E pisi* conidia was 200-300 per mm^2 on the pea leaves Conidial germination on healthy and treated pea leaves - was recorded after 24 and 48 h of treatment Statistical analysis was performed using Origin software

Results

Treatment with different concentrations (50,100,150 ppm) of L-phenylalanine increased activity of phenylalanine ammonia lyase (PAL) in comparison to control

(Fig 1) Pea leaves treated with 50 ppm L- phenylalanine, maximum PAL activity was observed after 24 h of treatment At 100-ppm concentration, PAL activity decreased linearly up to 96 h However, pea leaves treated with 150 ppm L-phenylalanine, PAL activity increased linearly and maximum activity was observed at 96 h (Fig 1)

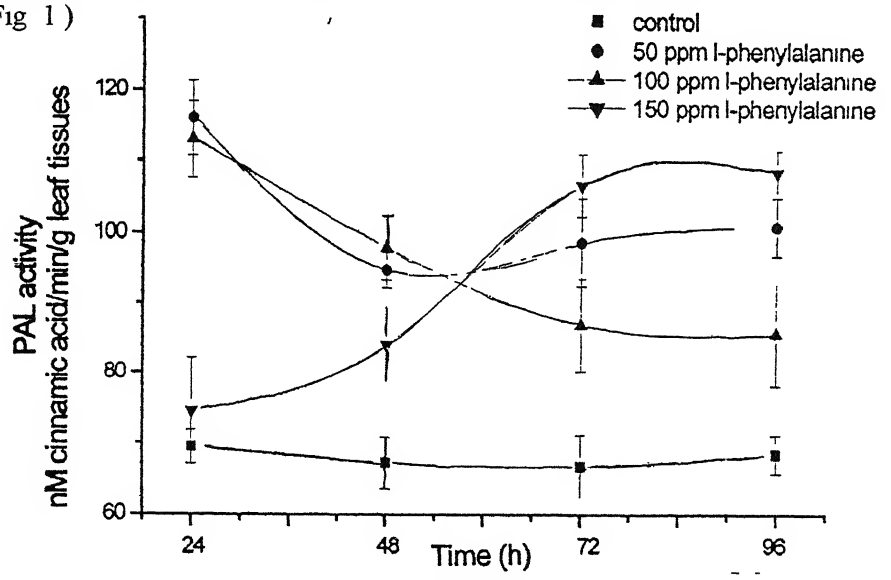

Fig 1– Effect of L-phenylalanine on PAL activity of pea leaves

Application of cinnamic acid also increased PAL activity but to a lesser extent compared to L-phenylalanine treatment (Fig 2) Maximum PAL activity was 95 8 nM cinnamic acid/min/g fresh wt after 24 h at 100 ppm Similarly, maximum PAL activity (95 3 nm cinnamic acid/min/g fresh wt) at 72 h was observed at 50 ppm cinnamic acid At 96 h, maximum PAL activity was observed at 50 ppm (83 6 nM cinnamic acid/min/g fresh wt) However, PAL activity was higher than their controls in all the concentrations

Treatment with both L-phenylalanine and cinnamic acid significantly reduced \ conidial germination of *E pisi* on pea leaves (Table 1) It is evident from the results that the concentrations of 100 and 150 ppm of both the compounds were equally effective in inhibiting conidial germination of conidia as compared to control It is evident from the results that exogenous L-phenylalanine treatment was more effective in reducing conidial germination as compared to cinnamic acid treatment on pea leaves

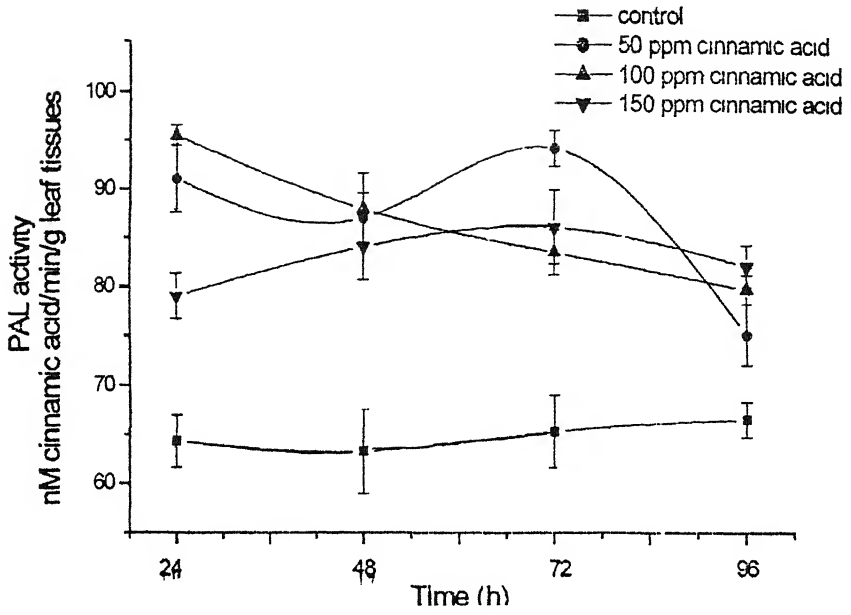

Fig 2– Effect of cinnamic acid on PAL activity of pea leaves

Table 1– Effect of L-phenylalanine and cinnamic acid on conidial germination (%) of *Erysiphe pisi* on pea leaves

Concentration (ppm)	Conidial germination (%)	
	Time (h) after inoculation 24 48	
	24	48
L-phenylalanine		
50	36±2 6	54±1 3
100	24±1 8	37±2 1
150	22±1 3	32±1 3
Cinnamic acid		
50	40±1 6	61±2 1
100	32±2 1	53±0 8
150	33±2 3	51±1 8
Control	52±1 5	68±1 5

Discussion

Plants may accumulate phenolics as a function of PAL activity as a means of passive defence[7] and the magnitude of the accumulation primarily depends on the supply of the primary precursor, L-phenylalanine[3] Foliar application of L-phenylalanine on pea leaves caused greater PAL activity as compared to untreated control is in conformity with the observations of Da Cunha[3] Increase in PAL activity is said to be circumstantial and also controversial It has been shown that cinnamic acid and their derivatives inhibit PAL activity while cinnamic acid was shown to be ineffective in sweet potato and pea[8] Our results indicate that higher concentration (150 ppm) of cinnamic acid increased PAL activity linearly till 72 h compared to control in pea leaves These results are in accordance to the results of Politycka[4] who demonstrated that cinnamic acid increased PAL activity and phenolic contents in cucumber

The result also indicate that exogenous application or both L-phenylalanine and cinnamic acid (one of the products of PAL) inhibited conidial germination on pea leaves, and thereby offered protection against pea powdery mildew disease Many studies indicate that greater accumulation of phenolics due to increased PAL activity, offered protection against disease and herbivory[2]

Acknowledgement

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Seasonal distribution of active ectomycorrhizal roots under *Cedrus deodara*

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Abstract

In this paper results are presented of two year study conducted on ectomycorrhizal active of *Cedrus deodara* considering the soil volume, soil moisture, pH and NPK content The ectomycorrhizal activity was maximum during rainy season in all the soil layers, Correlation between organic layer (humus) and ectomycorrhizal activity was also observed pH in the acidic range was observed to support maximum mycorrhizal activity in all soil layers during rainy season and humus was much acidic which supports maximum ectomycorhizae However, Nitrogen and Potassium were higher in litter and humus layer but Phosphorus was maximum in mineral soil It was also found that mycorrhizal activity was higher in soil with low phosphate content i e with Humus

(**Keywords** ectomycorrhizal roots/ *Cedrus deodara* /seasonal distribution)

Introduction

Mycorrhizal fungi are major component of soil microflora in any ecosystem. Environmental factors and soil conditions are known to influence he mycorrhizal association in a given ecosystem During the development of growth of natural forest, a number of changes in vegetation occur which, in turn affect the physico-chemical attributes of the soil in which the mycorrhizae are active Hence, in the present investigation, it was desired to estimate the mycorrhizal activity of roots of *C deodara*, Which is a reflection of their distribution in a natural ecosystem

Factors that influence the distribution of ectomycorrhizae are the physical condition of soil, temperature, moisture, interaction with other sol microbes and nutrient uptake etc.

The present study was specifically aimed at estimating the distribution and activity of mycorrhizae in roots of *Cedrus deodara* Different factors like soil moisture, pH and NPK contents were estimated in al three soil layers (litter, humus and mineral soil) during different seasons for the two consecutive years

Materials and Methods

The experimental site located behind Shimla city, is characterized with five distinct seasons-summer (May-June), rainy season (July-August) autumn season

(September-October), winter season (November-January) and spring season (February-April) Summer season is associated with moderate temperature and low humidity Rainy season is the wettest part of the year and receives about 55-70% of total annual rainfall, winter is very cold, snow and frost also occur in this season

Sampling technique Soil samples were taken with an "Impact soil sampler" having a cylindrical shape

Soil core was removed from near the vicinity of tree species Each core was sub divided in the field into base fractions litter, humus and mineral soil

Volume (cm^3) of different fractions was determined by measuring the depth of the layers Mycorrhizal root tips count was done following Harvey *et al*[1] Every layer of the soil was sieved through 25 mesh sieve and only active root tips were counted (The active root tips were swollen and brighter in colour than dead root tips which were dark and shriveled)

To determine percent moisture, fresh weigh of soil was taken initially and then was weighed again after drying it in over at 110 °C

For determining the pH, 5 gm of soil from each sample was dissolved in 25 ml of distilled water in ratio of 1 5 by Philips pH meter

Soil analysis for NPK content in different soil layer was done by following methods

For determine available P in soil, the Olsen's method[2] was used To observed nitrogen in litter layer of *C deodara* Kjeldahl method (Jackson, 1958)[3] was used To estimate nitrogen in humus and mineral soil, at first organic carbon (OC) was estimated by the help of Walkley and Black method[4] and after that nitrogen was observed by Subbiah and Asija method[5] and potassium was estimated by using a flame photometer

Results

The result of the study on distributional pattern of ECM in different soil layers in the root zone of *Cedrus deodara* are given below

The ECM count in the litter layer reveals the ECM root tips count/litter of litter layer of *C deodara* was minimum during Nov-Jan (31 0/liter and 29 4/liter) and maximum during Sept-Oct (82 3/litter) and July-Aug (77 2/litter) for two successive years respectively However, the ECM root tips were absent during May-June

The two year, study on litter layer of *C deodara* reveals that minimum volume of litter was during Nov-Jan (162 8 cm^3 and 164 8 cm^3) while maximum was during May-June (266 9 cm^3 and 262 9 cm^3) in both the year

The percent moisture of litter in the litter layer of *C deodara* was minimum during May-June (13 1% and 15 5%) and maximum during July-Aug (56 8% and 60 2%) in these two years respectively

The pH of litter layer of *C deodara* was minimum during July-Aug (6 2 and 6 1) and maximum during May-June (6 5 and 6 4) in the two years respectively

The percentage of nitrogen in litter layer of *C deodara* was minimum Nov-Jan (0 7% and 0 8%) in these two years and was maximum during July-Aug 1 3% in first year and during May-June of the second year it was 1 1%

The percentage of phosphorus in litter layer of *C deodara* was minimum during Nov-Jan (0 05% and 0 06%) and maximum during July-Aug (0 111% and 0 114%) in these two years respectively

The percentage of potassium of litter layers of *C deodara* was minimum during Nov-Jan (2 9% and 2 8%) and maximum during May-June (3 6%) in the first year and Sept-Oct (4 1%) in second year

The ECM root tips/liter in humus of humus layer of *C deodara* was minimum during Nov-Jan (126 8/liter and 128 8/liter) and maximum during July-Aug (406 7/liter and 405 1/liter) in first year and second year respectively

With regard to volume of humus in the humus layer *C deodara,* it was lowest during May-June (207 6 cm^3 204.7 cm^3, and highest during Sept-Oct (431 7 cm^3 and 431 7 cm^3) in both the years

The percent moisture of humus in humus layer of *C deodara* was minimum during May-June (16 3% and 18 6%) and maximum during July-Aug (41 4% and 41 7%) in first and second respectively

The pH values in the humus layer of *C deodara* were minimum during July-Aug (6 1) and during Feb-April (6 4) in first and second year respectively and maximum was during May-June (5 5 and 6 5) in both the years

The minimum percentage of nitrogen in humus layer of *C. deodara* was observed during Nov-Jan (0 23%) and during Feb-April (0 17%) in first and second year respectively and maximum percentage was observed during July-Aug (0 25% and 0 26%) in 50th the year

The minimum percentage of phosphorus in humus layer of *C deodara* was during Sept-Oct (1 40%) and during Nov-Jan (1 44%) in first and second years respectively and maximum during July-Aug (1 9% and 1 8%) in both the years

The minimum percentage of potassium in humus layer of *C deodara* was during Nov-Jan (3 01% and 3 2%) and maximum during July-Aug (3 4% and 3 6%) in first and second year respectively

The ECM root tips/litter in mineral soil of the mineral soil of *C deodara* was minimum during Feb-April (11 8/liter and 11 1/liter) and maximum during July-Aug (46 6/liter and 44 8/liter) in the first and second year respectively

The volume of mineral soil in the mineral soil layers was lowest during Sept-Oct (1703 9 cm^3 and 1705 8 cm^3) and highest during Feb-April (1913 3 cm^3) in first year and during May-June (1887 2%) in second year

The percentage of moisture of mineral soil layer was minimum during May-June (15 2%) in the first year and during May-June (13 9%) in the second year while it was maximum during July-Aug (34 9% and 34 9%) in both the years

The pH of mineral soil layer of *C deodara* was minimum during July-Aug (6 3 and 6 2) and maximum during May-June (6 7 and 6 6) in the second year respectively

The minimum percentage of nitrogen in mineral soil of *C deodara* was during Nov-Jan (0 22% and 0 21%) and maximum during July-Aug (0 24 and 0 23%) in first and second year respectively

The minimum percentage of phosphorus in mineral soil layer of *C deodara* was during Feb-April (1 6%) in first year and during Feb-April (1 687%) in first year and maximum during July-Aug (2 004% and 2 102%) in Is and IInd year respectively

The minimum percentage of potassium in mineral soil of *C deodara* was during Nov-Jan (2 9% and 3 0%) and maximum during July-Aug (3 5% and 3 5%) in first and second year respectively

The above observation were analyzed by One Way ANOVA test for different factors in different layers The F-ratio was significant during different seasons The DMRT (Duncans Multiple Range Test) was applied to find out the significance of difference during different seasons

Table 1– Minimum and Maximum values of ECM root tips/liter, volume of soil, moisture percentage,pH and NPK percentage in litter, humus and mineral soil during this para of two years

Soil Layers

		Ist year			IInd year		
		Litter	Humus	Mineral Soil	Litter	Humus	Mineral Soil
ECM root Tips/Liter	Min	31 000/liter Nov - Jan	126 881/liter Nov-Jan	11 844/liter Feb-April	29 444/liter Nov-Jan	128 889/liter Nov-Jan	11 125/liter Feb-April
	Max	82 375 /liter Sept - Oct	406 748/liter July-Aug	46 677/liter July-Aug	77 200 /liter July-Aug	405 167/liter July-Aug	44 833/liter July - Aug
Volume of Soil	Min	132 885 cm^3	207 632 cm^3	1703 979 cm^3	164 850 cm^3	204 753 cm^3	1705 811 cm^3
	Max	266 901 cm^3 Nov-Jan	432 532 cm^3 May-June	1913 300 cm^3 Sept-Oct	262 975 cm^3 Nov-Jan	431 747 cm^3 May-June	1887 272 cm^3 Sept-Oct
Moisture %	Min	13 180% May-June	13 3000% May-June	15 250% May-June	15 525% May-June	18 650 % May-June	13 973% May-June
	Max	56 850% July-Aug	41 480% July-Aug	34 950% July-Aug	60 220% July-Aug	41 700% July-Aug	34 950% July - Aug

Contd Table 1

Contd Table 1

	Min	6 250 July-Aug		6 190 July-Aug		6 343 July-Aug		6 180 July-Aug		6 460 July-Aug		6 271 July-Aug	
pH			–		*		*		*		–		–
	Max	6 533 July-Aug		6 547 July-Aug		6 795 July-Aug		6 460 July-Aug		6 596 July-Aug		6 672 July-Aug	
	Min	0 727% Nov-Jan		0 237% Nov-Jan		0 215% Nov-Jan		0 773% Nov-Jan		0 173% Nov-Jan		0 210% Nov-Jan	
N%			–		*		*		–		–		–
	Max	1 24% July-Aug		0 258% July-Aug		0240% July-Aug		1 135% July-Aug		0 267% July-Aug		0 236% July-Aug	
	Min	0 0511% Nov-Jan		1 404% Sept-Oct		1 6113% Feb-April		0 058% Nov-Jan		1 442% Nov-Jan		1 687% Feb-April	
P%			–		*		*		–		–		*
	Max	0 111% July-Aug		1 404% July-Aug		1 613% July-Aug		0 058% July-Aug		1 442% July-Aug		1 687% July-Aug	
	Min	2 944% Nov-Jan		3 052% Nov-Jan		2 977% Nov-Jan		2 833% Nov-Jan		3 257% Nov-Jan		3 011% Nov-Jan	
K%			–		*		*		–		*		–
	Max	3 67% May-June		3 463% July-Aug		3 536% July-Aug		4 083% Sept-Oct		3 628% July-Aug		3 535% July-Aug	

The significant and non-significant values between maximum and minimum values are given in Table 1 (Significance is shown by *)

Discussion

The observations made on the distribution of ectomycorrhizae in *Cedrus deodara* reveal that during the two years of study maximum litter was encountered during May-June This is supported by the fact that these months coincide with the denudation

The ECM count in litter layer was maximum during Sept-Oct followed by July-Aug in the first year during July-Aug in second year The moisture content in this layer was also maximum during this season This shows a close relationship between ECM count and moisture content Similar relationship in ECM count and moisture content was also recorded by Warley and Hasckalyo[6] corroborating the findings of the present study

Like litter the maximum ectomycorrhizal activity in humus and mineral soil was observed during rainy season when the, soil moisture was also maximum. During rainy season however the litter layer had more moisture content than humus and mineral soil. In the rest of the seasons it was the humus layer, which supported maximum moisture This is possibly because of the reason that humus layer has greater water holding capacity Hence it also shows greater ectomycorrhizal activity than the other two layers (Figs 1 and 2) The ability of soil organic reserves to support more ectomycorrhizal root activity has also been observed may scientists[7-12] They noticed very low number of ECM root tips in the mineral soil (4%) as compared to the humus layer (61%) in their studies They described that the low ECM activity in mineral soil is due to low moisture content and low organic content

The litter layer also supported low amount of ECM activity in comparison to humus (Figs 1 and 2) Harvey *et al.*[12] also stated that less activity in litter is probably due to its extreme variability, hot and dry during summer and near freezing during winter Harini Kumar and Bagyaraj[13] also recorded similar observation in mango and leucaena stating that the summer months are unfavorable for mycorrhizal activity

Since the moisture content during winter season and summer season was low, the distribution of ectmycorrhizal root tips seems to have decreased during wither season Muttiah[14] and Warley and Hasckaylo[6] have also shown that reduced soil moisture can change mycorrhizal relationship

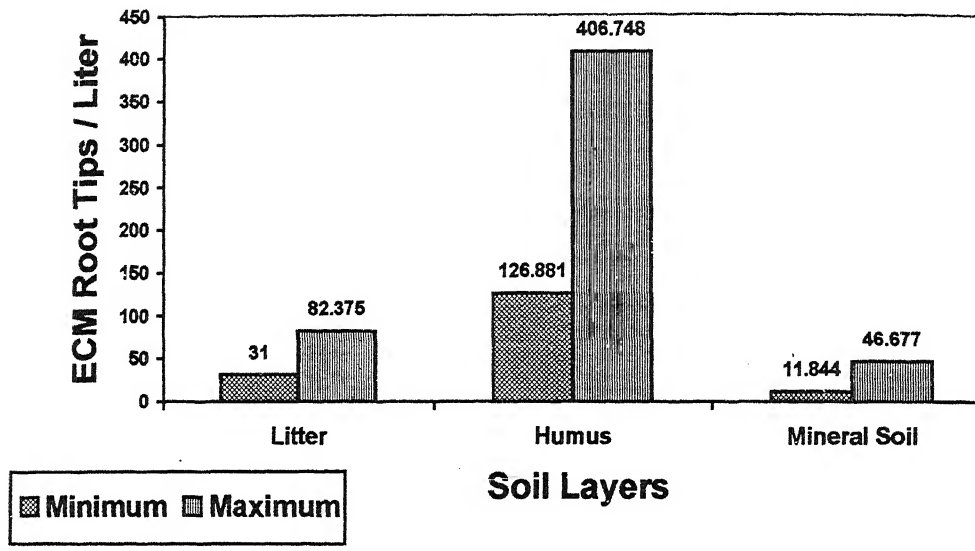

Fig. 1– Minimum and maximum number of active ectomycorrhizal tips/ litter in each soil layers in Ist year.

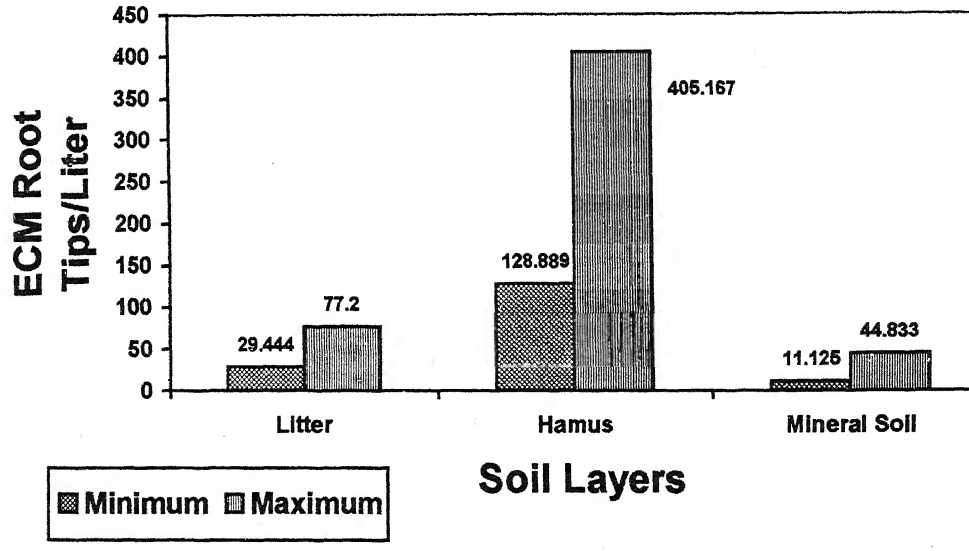

Fig. 2– Minimum and maximum number of active ectomycorrhizal tips/litter in each soil layers in IInd year.

Marx and Zak[14] observed that the mycorrhizal activity is more when pH is in the acidic range In the present study ECM activity was maximum during rainy season in all the three soil layer i e , litter, humus and mineral soil when the pH of soil was much acidic compared to other season is in consonance with the finding of Marx and Zak[14]

The humus layer was more acidic than the mineral soil layer and it also exhibited maximum ECM activity throughout the year These observations are supported by Marais and Kotze[15] They studied the effect of pH on mycorrhizal development in *Pinus patula* and observed that acidic pH positively influences the mycorrhizal activity, which has further been supported by Lawrence[15] Erland and Soderstorm[17] repeated that seedlings grown in a forest humus soil about a pH gradient from 4 to 7 5 showed increased proportion of mycorrhizal root tips form 70% at pH 4 to barely 100% around pH 5 and then decreased to below 40% at pH 7

There are seasonal variations in the availability of nutrients in natural ecosystem soil (Gupta and Rorison[19], Versogloa and Fitter[20]) In the present study the variation in between seasons in all there soil layer shows differences The NPK content in soil layers was maximum during rainy season The NPK was maximum when the mycorrhizal activity was also maximum The nutrient content in soil seem to be directly related to ECM activity and both these are influence by sufficient moisture and acidophilic pH of the soil

When all three soil layers are compared it was observed that nitrogen and humus have more N and K than the mineral soil of chir pine while phosphorus was maximum in the mineral soil Dighton and Coleman[20] illustrated that P-fixing capacity of soil was greater in the mineral soil than the organic component

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Anti-fungal activity of *Bacillus thuringiensis* var *kurstaki* on *Trichoderma viride*

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Abstract

Trichoderma viride and *Bacillus thuringiensis* have gained a prominent stature in Integrated Pest Management (IPM) for the control of a broad spectrum of plant pathogenic fungi and several classes of insect pests respectively During our experimental work, it was discovered that the growth of *T viride* was adversely affected when plated along with *B thuringiensis* var *kurstaki* The antagonistic nature of *B. thuringiensis* towards *T viride* poses a question mark that whether these two organisms could be applied simultaneously in fields or not Further investigations related to the nature and biochemistry of this inhibitory molecule are in prospective to determine the exact biochemical steps involved in this inhibition

(**Keywords** : *Trichoderma viride* / inhibition / *Bacillus thuringiensis)*

Introduction

With the gradual modernization of agriculture, the pest problem has also become complex as it is generally admitted that the inevitable corollary of intensive agriculture is the proportionate accentuation in the intensity of pest attack Integrated Pest Control has been the conceptual basis for most modern plant protection measures It developed in response to the evidence of the failure of pesticides due to the development of resistance, in the targeted species *Trichoderma viride* and *Bacillus thuringiensis* have gained a prominent stature in IPM for the control of a broad spectrum of plant pathogenic fungi and several classes of insect pests respectively. *T viride* is a potent organism serving from decades in biological control because it decreases or eliminates allelopathic chemicals, prevents the allelopathic effects of VAM fungi[1], increases acidification of soil thereby chances of pathogenic fungi are reduced due to antagonistic interactions and inhibits the growth of true 'dry rot fungus' *Serpula lacrymans,* which is the most significant fungal cause of damage to timber in built environment in many temperate regions of the world[2]. *B thuringiensis* is the

most promising candidate amongst all available bacterial insecticides, which has been successfully produced commercially and marketed in some of the most developed countries of the world because it is readily available in nature and can be easily isolated, does not have significant history of mammalian pathogenecity[3] and because of its broad spectrum of toxins that makes it highly potent to act on several insect pathogens

In the present investigation an antagonistic nature of *B thuringiensis* towards *T viride* is reported, which poses a question mark that whether these two organisms could be applied simultaneously in fields or not!

Materials and Methods

Inoculum

The laboratory fungal culture *T viride* (BCF-18) and the bacterial culture *B thuringiensis* var *kurstaki* (BCB-9) that were maintained on Sabouraud Dextrose agar (SDA) or broth (SD) (peptone 10 G, Dextrose 40 G, Agar 20 G and Distilled water 1000 mL) and a semi synthetic medium (Glucose 0 1 G, Yeast Extract 0 2 G, Amm Sulphate 0 2 G, K_2HPO_4 0 5 G, NaCl trace and Distilled water 100 mL)[4] respectively were used in the present study

Detection of fungicidal activity by the deferred agar spot test (DAS)

The fungicidal activity was checked both on SDA and semi synthetic agar medium The method given by Tagg *et al*[5] was followed 500 µl culture of *B thuringiensis* grown for 48 h in semi synthetic broth specified earlier, was inoculated with SDA and semi synthetic agar medium respectively, by the pour plate technique in triplicates After solidification of the medium, the mycelial mats of *T viride* grown on SDA plates at 25 ± 1°C for three days were cut into round agar plugs using a 5-mm-diameter cork borer was inoculated on its surface. Plates were incubated at 25 ± 1°C and after three days, observations were made on the colony diameter of *T viride,* as a measure of mycelial growth, following the method of Cagan and Uhlik[6] The measurements were recorded on three or more replicates

Antifungal activity at different glucose concentrations

Semi synthetic medium with different concentrations of glucose (0 1 %, 0.5% and 1%) were prepared, and plated with 500 µl of 48 h grown *B. thuringiensis* var *kurstaki* culture by the pour plate technique in triplicates. After solidification of the medium,

the mycelial mats of *T viride* grown on SDA plates at 25 ± 1°C for three days were cut into round agar plugs using a 5-mm-diameter cork borer was inoculated on its surface Plates were incubated at 25 ± 1°C and after three days, observations were made on the colony diameter of *T viride,* as a measure of mycelial growth, following the method of Cagan and Uhlik[6] The measurements were recorded on three or more repetitions Semi synthetic agar medium with the mentioned concentrations of glucose and which was not inoculated with *Bacillus thuringiensis* culture were used for controls

Results and Discussion

Anti-fungal activity

Antagonism is a phenomenon in which one microorganism affects the growth of other microorganism by severely affecting its environment From the results presented in Fig 1 and 2, it was evident that the growth of *T viride* was normal on SDA and semi synthetic agar medium but was inhibited in the presence of the bacterium *B thuringiensis* var *kurstaki* An inhibited growth of *T viride* was seen in the test plates (Fig 2) when compared with the control plates where no bacterial culture was present After three days of incubation there was green coloured pigmentation and conidiation in the control plates where as in the test plates growth was completely inhibited at 0.5 cm diameter and no further growth of the fungus was observed

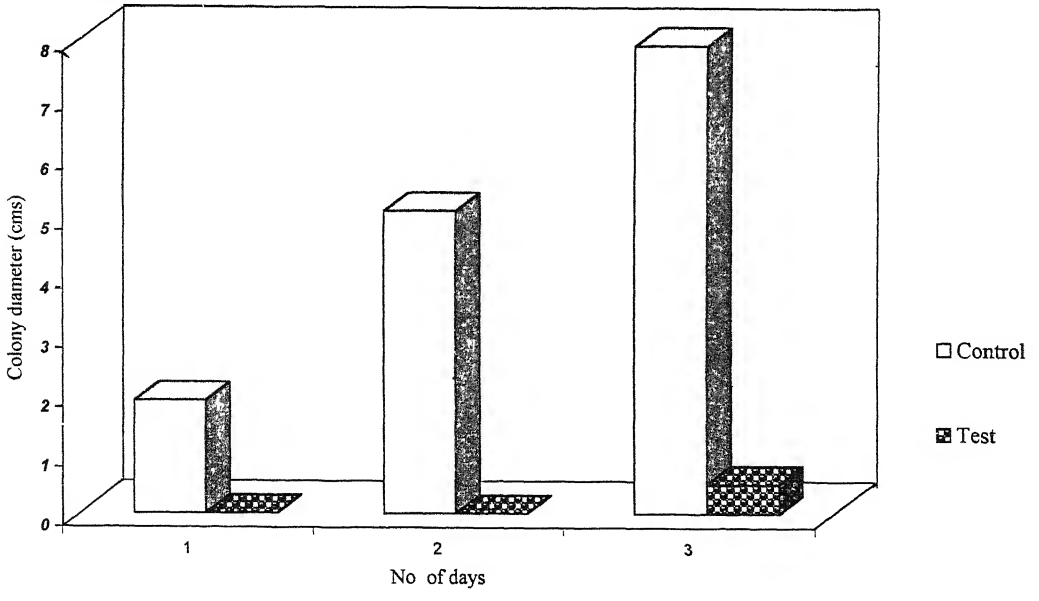

Fig 1– Anti-fungal activity of *B thuringiensis* var *kurstaki* towards *T viride* grown on SDA medium

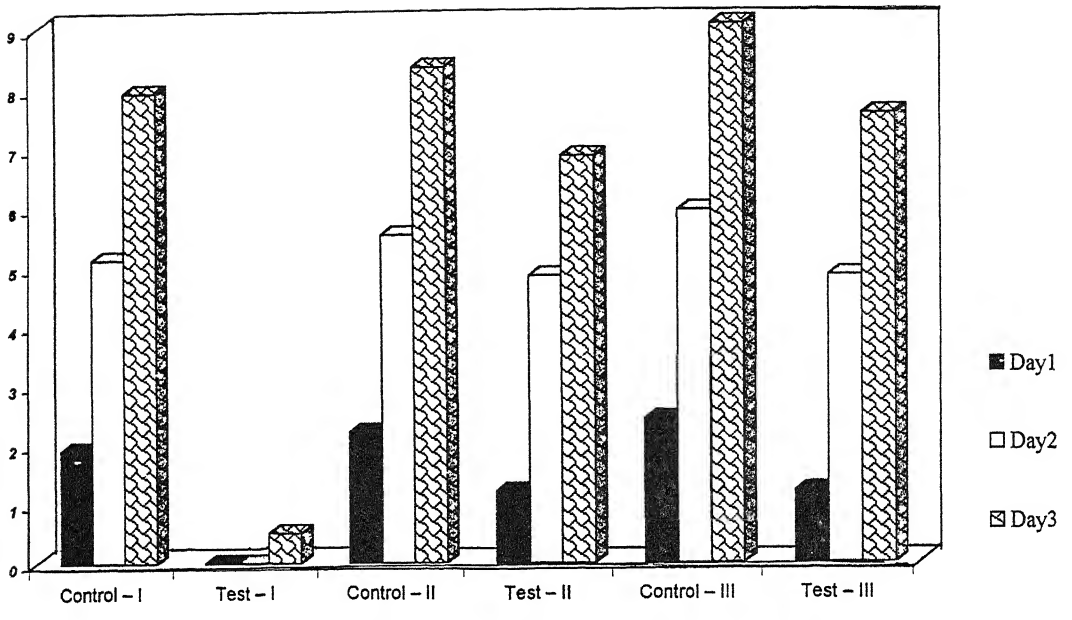

Fig 3– Effect of *B thuringiensis* var *kurstaki* and different concentrations of sugar used in the semi synthetic medium on the mycelial growth of *T viride*

Microorganisms have economic importance for a multitude of reasons There are many possibilities of adding new organisms to the list of economically important microorganisms by selecting wild strains that might produce new bio-molecules The strike back of pathogens has revitalized the search for new bio-molecules and compounds[7] From the results presented, the antifungal activity of bacterial strain was evident and may be accepted because, it is well known that most, if not all, bacterial species are capable of producing a heterogenous array of molecules in the course of their growth *in vitro* (and presumably also in their natural habitats) that may be inhibitory to other organisms[5] Klaenhammer[8] and Jack *et al*[9] have reported that bacteriocins are polypeptide antibiotics that can pocess bacteriocidal, fungicidal, metal-chelating and immuno-modulating activities and that they are frequently found as secondary metabolites produced by various microorganisms, such as Gram-positive bacteria of the genus *Streptomyces,* lactic acid bacteria and genus *Bacillus* Although it is agreed that *B thuringiensis* is not virulent and invasive against fungal

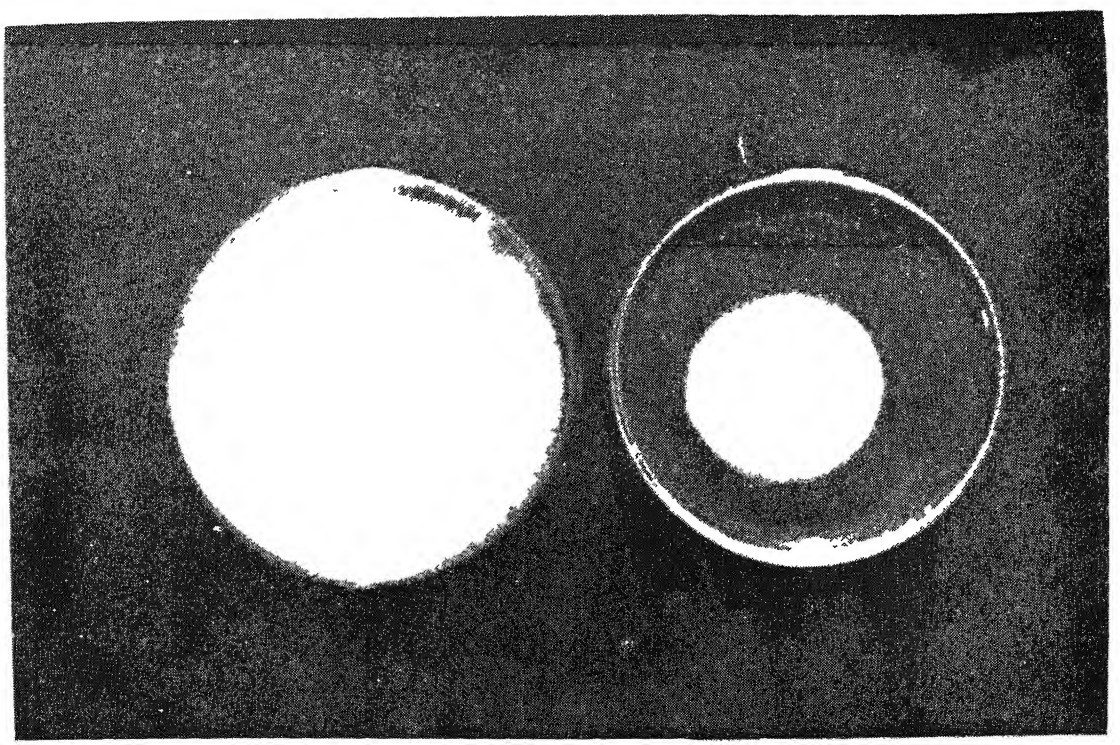

Fig 2– Photograph representing inhibition of *T viride* by *B thuringiensis* var *kurstaki*

pathogens[10], but in our case it was definitely showing antagonism against *T viride in vitro,* which indicates an ecological imbalance Similar antifungal activity of the bacterial strains has been studied by Marten *et al* [11]

Antifungal activity at different glucose concentrations

Fungi being saprophytic or parasitic require an external source of nutrition Most of the fungi require an external source of carbon (C), hydrogen (H), oxygen (O) and nitrogen (N) The first of three principle elements are provided by carbohydrates, while nitrogen source may be organic or inorganic Among different sources of carbohydrates, glucose is the preferred one for nearly every fungus[4] The enzyme system of fungi is well developed to synthesize other essential requirements from these basic elements Since glucose is the preferred source of carbon for every fungi, it has been observed in this experiment that the growth of *T viride* was enhanced as the concentration of glucose in the semi synthetic medium was increased from 0 1% to 0 5% and 1% (Fig 3) But under similar conditions the presence of *B thuringiensis* var *kurstaki* inhibited the growth of the fungus Thus, it can be concluded that the bacterium by itself is responsible for this inhibition of the growth of *T viride*

Therefore, the results presented indicate that *B thuringiensis* has an antagonistic activity towards the growth of *T viride* Although it is agreed that *B thuringiensis* is not virulent and invasive against mammalian pathogens, it is definitely showing antagonism against *T viride,* which indicate a negative ecological compatibility A similar case was observed by Kim *et al* [10] and Sinha and Basuchoudhary[12] wherein, actinomycetes antagonizes various pathogenic fungi in vitro as well as in vivo

Non-selectivity problems in pest and pathogen control have lead to an alternative arrangement of integrated pest control Although, it has been stated that simultaneous or sequential application of the pathogens in integrated control of insect pests involve a variety of mechanisms that act together causing high mortality, but this is the case only against a single targeted pest In such a case, the targeted pest is counter-attacked by both the pathogens simultaneously making it highly susceptible and thereby killing it Therefore, it can be argued that the integration of two pathogens has been very effective, as both the pathogens are ecologically non-disruptive control agents in view of their relative specificity But in cases where two different biological control agents are used against two different targeted pest it is necessary that one biological control agent is not interfering with the other biological control agent's environment and if this is the case, these agents cannot be used in integration

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Misidentification of Aeromonads with *Vibrios* and *Chromobacterium* species isolated from fresh water environments

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Abstract

Among 157 bacterial species considered as aeromonads, isolated from river Narmada, at Jabalpur (MP), India, during August 96 to August 98, only 119 isolates were confirmed as aeromonads, 28 and 10 isolates were confirmed as *Vibrio* and *Chromobacterium* spp respectively The present study advocates the application of modern trends for the identification of aeromonads apart from the commercial methods

(**Keywords** Aeromonads/ *Vibrios/ Chromobacterium*)

Introduction

The accurate and definitive identification of microorganisms, including bacteria is one of the cornerstones forming the joint foundation of the fields of microbiology and infectious diseases Between 1980 and the end of 1996, there was a 238% increase in the total number or approved names in the literature, with the number rising to 5,569 taxa (Euzeby, 1997)[1]

Aeromonads forms a large group of bacteria including different species and subspecies. The turbulent changes in the taxonomy witnessed over the past 10 years is referred as 'sea of change' by Carnahan (1993)[2] Kannan and Nair (2000)[3] and Nair and Carnahan (2003)[4] showed the distribution of recognized species of *Aeromonas* within 14 hybridization group. The genus currently comprises of 17 species (*A hydrophila, A. bestiarum, A. salmonicida, A caviae, A. media, A. eucrenophila, A sobria, A. jandaei, A veronii, A schubertii, A trota, A allosaccharophila, A. enchleia, A. popoffii, A. culicicola, A enteropelogenes* and *A icthiosmia*), however, taxonomy of the group is not yet resolved

Aeromonads are cosmopolitan in distribution, still aquatic environments represents largest pool of this organism Therefore, source of aeromonads infection is generally considered as of aquatic origin, which proves to be potential water borne pathogen

Despite the taxonomic advances, enumeration of aeromonads possesses Herculean challenges to the microbiologists Commercial identification systems have often been unable to identify aeromonads to the species level or as part of the complex Often *Aeromonas* species are mistakenly identified as vibrios, with which they share many phenotypic characteristics (Janda *et al* 1995)[5] A common example of this misidentification involves *A caviare* being misidentified as *V fluvialis* (Janda *et al* 1995)[5]

Abbott *et al* (1998)[6] reported the misidentification of *A schubertii* and *A veronii* biotype veronii as *Vibrio damsela* and *Vibrio cholerae* respectively In the first case, the patient was suffering from diarrhea, with a history of liver cirrhosis, while in the other case the patient needed an immediate cholecystectomy, the cultures of the infected gall bladder yielded a gram-negative bacillus, presumptively identified as *V cholerae* The identifications were based upon the API 20E (API bioMerieux, Hazelwood Mo) It is needless to say that, had the isolated organisms be screened with the key biochemical reactions that separate Vibrionaceae from Aeromonadaceae, the misidentifications could have been avoided

The misidentification of *Aeromonas* as vibrios could unnecessarily panic the health care workers and professionals because of the public health significance of vibrios

Thus, during the present study, an environmental survey was carried out to assess the problem of misidentification of *Aeromonas* isolated from the largest west flowing river of India, the Narmada

Materials and Methods

Four major sampling stations, *viz* Tilwaraghat, Gwarighat, Jelharighat and Bheraghat were selected, as these are surrounded by urbanized catchment areas The water at these sampling stations is used for drinking, washing, bathing, cattle wading, irrigation, recreational and aesthetic purposes Sampling was carried out from August 96 to August 98 at monthly intervals from all the four sampling stations.

2000ml of water samples were collected and isolation and enumeration of aeromonads was done following the membrane filtration technique (Rippey and Cabelli, 1979, APHA, 1985)[7,8]

Identification of aeromonads was done following morphological, biochemical and cultural characteristics as described by Altwegg *et al* (1990)[9] Borell *et al* (1998)[10] and with the help of Bergey's manual of systematic bacteriology[11] and PIB Computer Kit (1993)[12]

Results and Discussion

157 bacteria were isolated using specific selective and enrichment media prescribed for the isolation of aeromonads from environmental sources following the method of Rippey and Cabelli (1979)[7] and Arcos *et al* (1988)[13] on the basis of mannitol fermentation, oxidase reaction and resistance to vibriostatic agent 0/129 Out of 157, only 129 bacterial isolates were considered as *Aeromonas* spp on the basis of biochemical tests as proposed by Borell *et al*, 1998 [10] (Table 1)

The remaining 28 isolates other than the *Aeromonas* were identified as *Vibrio* spp (Table 2). Presumptive aeromonads were subjected to specific biochemical tests to identify them up to the genomospecies level Only 110 were confirmed as *Aeromonas* (belonging to 8 different genomospecies) whereas ten isolates which gave unusual results were identified as *Chromobacterium violaceum* (Table 4) and nine isolates remained unidentified up to the species level, having some unusual characteristics (Table 3)

Misidentification of aeromonads as vibrios is still a continuing problem. The oxidase positive non-pigmented strains of *Chromobacterium violaceum* could be misidentified as *Vibrio* or *Aeromonas* species This will lead to improper treatment of diseases in the absence of proper diagnosis of the causal organism. Moreover, the failure of commercial systems for confirmative identification of pathogenic microbes is a cause of concern for clinicians and microbiologists

However, this concern is only the tip of an iceberg of a potentially larger problem with more important ramifications With the advent of the availability of commercial systems for rapid analysis, the number of case reports linking old agents to new diseases and new or unusual (rare) agents to infectious processes has risen dramatically Reliance on the commercial identification systems can lead to misidentification, which can translate into an inaccurate body of information in the

medical literature concerning the clinical significance of many microbial species (Janda and Abbott 2002)[14]

For the present study, river Narmada was selected since, it's a very important source of fresh water supply in the city of Jabalpur and huge slums of populations are dependent upon the river water for variety of day to day activities, all year round Any misidentification and subsequent misdiagnosis and treatment could be very dangerous

Table 1– Biochemical tests for the presumptive identification of aeromonads isolated from river Narmada at Jabalpur (August 96 to August 98)

S No	Test for identification	Aeromonads (129)	Other genera (28)
1	Gram stain	-	-
2	Shape	Rods	Rods
3	Oxidase test	+	-
4	Growth in Nutrient broth at 37°C	+	+
5	Growth in Nutrient broth at 3 7°C-0% NaCl	+	-
6	Growth in Nutrient broth at 37°C-6% NaCl	±	+
7.	Glucose fermentation (TSI)	+	+
8	Acid from Inositol	-	+

The present study provided insights into the consequences of the misidentification of *Aeromonas* spp and also suggests synergistic use of biochemical tests and modern methods based on DNA-DNA hybridization, RFLP of PCR amplified 16S rRNA genes, ribotyping, to accurately identify this enigmatic group of pathogen

Table 2– Confirmative Identification of 28 bacterial isolates presumptively identified as Aeromonads isolated from river Narmada at Jabalpur (August '96 to August '98)

S No	Mor Cha		Cul Cha	Biochemical characteristics																	
	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v
1	C	R	-ve	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	+	+	-
2	C	R	-ve	+	+	+	+	+	+	+	-	+	-	-	+	+	-	-	-	-	-	-
3	C	R	-ve	+	+	+	+	+	+	+	-	+	-	-	+	+	-	-	-	-	-	-
4	C	R	-ve	+	+	+	+	+	+	-	-	+	-	-	-	+	+	+	-	-	-	-
5	C	R	-ve	+	+	+	+	+	+	+	-	+	-	-	-	+	-	-	-	+	+	-
6	C	R	-ve	+	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	+	+	-
7	Y	R	-ve	+	+	+	+	+	+	-	-	-	-	-	+	+	-	-	-	+	+	-
8	Y	R	-ve	+	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-
9	C	R	-ve	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	+	+	-
10	C	R	-ve	+	+	+	+	+	+	-	-	+	-	-	-	+	+	+	-	-	-	-
11	C	R	-ve	+	+	+	+	+	+	-	-	+	-	-	-	+	+	+	-	-	-	-
12	C	R	-ve	+	+	+	+	+	+	-	-	+	-	-	-	+	+	+	-	-	-	-
13	Y	R	-ve	+	+	+	+	+	+	-	-	-	-	-	+	+	-	-	-	-	-	-
14	Y	R	-ve	+	+	+	+	+	+	-	-	+	-	-	+	+	-	-	-	-	-	-

Table 2 Contd

Table 2 Contd

15	D	R	-ve	+	+	+	+	+	+	-	-	+	-	-	-	+	+	+	-	-	-	-
16	Y	R	-ve	+	+	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-
17	C	R	-ve	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	+	+	-
18	D	R	-ve	+	+	+	+	+	+	-	-	-	-	-	+	+	-	-	-	-	-	-
19	Y	R	-ve	+	+	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-
20	C	R	-ve	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	+	+	-
21	C	R	-ve	+	+	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-
22	C	R	-ve	+	+	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-
23	Y	R	-ve	+	+	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-
24	Y	R	-ve	+	+	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-
25	C	R	-ve	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	+	+	-
26	C	R	-ve	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	+	+	-
27	C	R	-ve	+	+	+	+	+	+	-	-	+	-	-	+	+	-	-	-	-	-	-
28	Y	R	-ve	+	+	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-

Mor Cha - Morphological characteristics, Cul Cha - Cultural Characteristics, a Pigment, b Shape, c Gram reaction, d Motility, e Fermentative, f Growth at 37°C, g Catalase, h, Oxidase, i, Gelatinase , j, H_2,S Paper, k, Arginine, l Amylase, m MR, n VP, o, Indole, p, Acid from Glucose, q Acid from Lactose, r Acid from Sucrose, S Gas production, t H_2S TSI, u, Simmon's citrate, v Urease, Y - Yellow, C - Cream, D - Dull, R – Rod

1 *V furnissi*, 2 *V vulnificus*, 3 *V vulnificus*, 4 *V metschnikovii*, 5 *V vulnificus*, 6 *V vulnificus*, 7 *V vulnificus*, 8 *V vulnificus*, 9 *V furnissi*, 10, *V metschnikovii*, 11 *V metschnikovii*, 12 *V metschnikovii*, 13 *V vulnificus*, 14 *V vulnificus*, 15 *V metschnikovii*, 16 *V vulnificus*, 17 *V furnissi*, 18 *V vulnificus*, 19 *V fulnificus*, 20 *V furnissi*, 21 *V vulnificus*, 22 *V metschnikovii*, 23 *V vulnificus*, 24 *V vulnificus*, 25 *V. furnissi*, 26 *V furnissi*, 27 *V vulnificus*, 28 *V vulnificus*

Table 3– Characteristics of Aeromonads not identified upto the genomospecies level

S No	L/O/A	BEH	GAS	VP	A/M/S	RAM	SOR	SOR 30°C	SALI	LAC	CIT	CIT 30°C	MAN	CEI
1	+/-/-	+	+	+	-/+/+	-	-	+	-	-	-	-	-	-
2	+/-/-	+	-	-	-/-/+	-	-	-	-	-	-	-	-	-
3	-/-/-	-	+	-	+/-/+	-	-	-	±	+	-	-	±	-
4	-/-/±	-	-	-	-/-/+	-	-	-	+	+	+	+	-	-
5	-/+/+	-	-	-	-/-/+	-	-	+	+	+	+	+	±	-
6	-/±/±	-	-	-	-/-/+	-	-	-	+	+	+	-	±	-
7	+/-/-	-	-	-	+/-/-	-	-	-	+	+	+	-	±	-
8	-/-/-	-	+	-	±/-/+	-	-	-	-	+	+	-	-	±
9	-/+/+	-	-	-	-/-/+	-	-	-	+	+	+	+	-	-

L/O/A, Lysine decarboxylase/ornithine decarboxylase/arginine dihydrolase, BEH Bile esculin hydrolysis, GAS, gas production from TSI, VP, Voges Proskauer test, A/M/S, acid from arabinose/acid from mannitol/acid from sucrose, RAM, acid from D-Rhamnose, SOR, acid from D- sorbitol; SALI acid from salicin, LAC, acid from lactose, CIT utilization of citrate, MAN, acid from mannose, CEL, acid from cellobiose, SOR 30°C, acid from D-Sorbitol at 30°C CIT 30°C, utilization of citrate at 30°C

Table 4– Confirmative Identification of bacterial isolates upto species initially identified as *Aeromonas* at generic level

S No	L/O/A	BEH	GAS	VP	A/M/S	RAM	SOR	SOR 30°C	SALI	LAC	CIT	CIT 30°C	MAN	CEI
1	-/-/-	-	-	-	-/-/-	+	+	-	-	-	+	-	+	-
2	-/-/-	-	-	-	-/-/-	-	+	-	-	-	+	-	+	-
3	-/-/-	-	-	-	-/-/-	-	+	-	-	-	+	-	±	-
4	-/-/-	-	-	-	-/-/-	±	+	-	-	-	-	-	-	-

Table 4 Contd

Table 4 Contd

5	-/-/-	-	-	-	-/-/-	±	±	-	-	-	-	-	+	-
6	-/-/-	-	-	-	-/-/-	-	-	-	-	-	+	+	+	-
7	-/-/-	-	-	-	-/-/-	-	±	-	-	-	-	-	+	-
8	-/-/-	-	-	-	-/-/-	±	-	-	-	-	-	-	-	-
9	-/-/-	-	-	-	-/-/-	-	+	-	-	-	+	-	-	-
10	-/-/-	-	-	-	-/-/-	+	-	-	-	-	-	-	+	-

L/O/A, Lysine decarboxylase/ornithine decarboxylase/arginine dihydrolase, BEH, Bile esculin hydrolysis, GAS, gas production from TSI, VP, Voges Proskauer test, A/M/S, acid from arabinose/acid from mannitol/acid from sucrose, RAM, acid from D-Rhamnose, SOR, acid from D- sorbitol, SALI, acid from salicin, LAC, acid from lactose, CIT, utilization of citrate, MAN, acid from mannose, CEL, acid from cellobiose, SOR 30°C, acid from D-Sorbitol at 30°C CIT 30°C, utilization of citrate at 30°C

Note All the isolates were identified as *Chromobacterium violaceum*

Acknowledgements

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Correlation studies in sorghum (*S. bicolor* (L.) Moench)

Received April 10, 2003, Revised July 12, 2004, Accepted August 28, 2004

Abstract

A set of fortyone elite genotypes of sorghum, was used for estimating correlation among fourteen morphological characters The study showed that leaf length was positively and significantly correlated with both green fodder yield as well as dry matter yield The plant height was significantly and positively correlated with dry matter yield

(**Keywords** correlation/sorghum)

For proper exploitation of the available variability the primary objectives must be to identify and select superior genotypes with desirable trait from a broad array of breeding material To accomplish this, the knowledge of inter relationship of yield and yield components is a prerequisite To analyse the extent of mutual relationship among different characters, study of correlation coefficient would be quite beneficial in formulating a suitable selection criteria This information may be used in predicting the correlated response to directional selection as well as in practicing indirect selection

The experimental materials consisted of fortyone elite genotypes (tillering and non tillering types) of sorghum were grown during Kharif 1998 at the G B Pant University of Agriculture and Technology, Pantnagar, in randomized complete block design with three replications Experiment was sown in a 6 row plot of 6m length with row to row spacing of 25 cm and plant to plant distance of 10 cm Observation on fourteen characters viz , days to 50 per cent flowering, plant height (cm) number of leaves/plant, leaf length (cm), leaf width (cm), total leaf area (cm^2), number of nodes/plant, internodal length (cm), stem diameter (cm) total soluble solids (T S S %), shootfly infestation (%), crude protein (%), dry matter yield (kg) and green fodder yield (kg) were recorded for evaluation of genotypes for intercharacter correlation The correlation between all possible pairs of characters under study, at genotypic, phenotypic and environmental levels were estimated according to Searle(1961).

The inter-character correlations at genotypic, phenotypic and environmental levels are presented in the Table 1

Table 1– Phenotypic, genotypic and environmental correlation coefficient among various characters in sorghum genotypes

S No	Characters		Plant height (cm)	No of leaves/ plant	Leaf length (cm)	Leaf width (cm)	Total leaf area $(cm^2)^2$	No of nodes/plant	Inter nodal length (cm)	Stem diameter (cm)	T S S %	Shootfly infestation (%)	Protein (%)	Dry matter yield (q/ha)	Green fodder yield (q/ha)
		rp	0 439**	0 204	-0 561**	0 096	-0 086	0 345*	0 338*	0 598**	0 752**	0 337*	0 396*	-0 299	-0 810**
1	Days to 50% flowering	rg	0 469	0 308	-0 691	0 119	-0 108	0 599	0 486	0 656	0 827	0 341	0 439	-0 308	0 844
		re	0 049	0 109	-0 163	-0 048	-0 067	0 059	0 087	0 036	-0 199	0 082	0 183	0 050	-0 011
		rp		0 543**	-0 097	-0 279	-0 028	0 516**	0 614**	0 457**	0 300	0 236	0 234	0 402**	-0 325*
2	Plant height (cm)	rg		0 806	-0 116	-0 338	-0 056	0 825	0 910	0 540	0 348	0 253	0 292	0 424	-0 354
		re		0 234	-0 046	-0 058	0 054	0 266	0 095	-0 087	-0 044	-0 027	-0 076	0 123	-0 009
		rp			-0 058	0 039	0 437**	0 942**	-0 018	0 201	0 159	0 111	0 184	0 215	-0 113
3	Number of leaves/plant	rg			0 028	0 006	0 413	0 976	0 535	0 438	0 265	0 241	0 338	0 334	-0 188
		re			-0 156	0 087	0 471	0 928	-0 447	-0 170	0 008	0 081	-0 015	0 044	0 015
		rp				0 047	0 379*	-0 185	-0 099	-0 159	-0 477**	-0 166	-0 212	0 226	0 478**
4	Leaf length (cm)	rg				-0 112	0 353	-0 198	0 239	-0 143	-0 663	-0 197	-0 295	0 318	0 685
		re				0 088	0 422	-0 193	0 073	-0 223	0 065	-0 122	-0 010	-0 203	-0 332
		rp					0 772**	0 108	-0 231	0 102	0 083	-0 016	0 033	-0 515**	-0 165
5	Leaf width (cm)	rg					0 799	0 141	-0 371	0 069	0 144	-0 022	0 090	-0 618	-0 224
		re					0 747	0 090	-0 046	0 224	-0 143	0 036	-0 136	-0 055	0 124
		rp						0 402**	-0 229	0 086	-0 081	0 005	0 027	-0 199	0 053
6	Total leaf area $(cm)^2$	rg						0 423	-0 253	0 151	-0 109	0 008	0 093	-0 258	0 076
		re						0 408	-0 205	-0 069	-0 020	-0 013	-0 114	-0 068	-0 011

	rp	-0 015	0 269	0 274	0 210	0 216	0 081	-0 250
7 Number of nodes/plant	rg	0 579	0 574	0 518	0 370	0 213	0 246	-0 446
	re	-0 403	-0 093	0 003	0 018	-0 003	0 022	-0 030
	rp		0 367*	0 175	0 173	0 13	0 187	-0 270
8 Inter nodal length (cm)	rg		0 588	0 308	0 267	0 213	0 246	-0 446
	re		0 056	-0 075	-0 082	-0 047	0 149	0 133
	rp			0 427**	0 185	0 275	-0 106	-0 378*
9 Stem diameter (cm)	rg			0 544	0 207	0 353	-0 126	-0 451
	re			-0 225	-0 062	-0 055	0 084	0 196
	rp				0 154	0 235	-0 258	-0 662 **
10 T S S (%)	rg				0 163	0 304	-0 214	-0 723
	re				0 099	-0 081	-0 126	-0 151
	rp					0 280	-0 011	-0 258
11 Shootfly infestation (%)	rg					0 319	-0 012	-0 265
	re					0 168	0 017	-0 124
	rp						0 094	-0 269
12 Protein %	rg						0 100	-0 303
	re						0 083	-0 081
	rp							0 480**
13 Dry matter yield (q/ha)	rg							0 475
	re							0 593

* Significant at 5% level ** Significant at 1 % level

At the phenotypic level, positive and significant correlation with green fodder yield was observed for leaf length and dry matter yield (Table 1) This was found to be supported by Sidhu and Mehndiratta[1] and Ahluwalia and Solomon[2]

The green fodder yield had negative correlation with shootfly infestation, protein%, and plant height The negative correlation of plant height with green fodder yield observed in the present study both at genotypic and phenotypic levels is in contrast to earlier findings (Singhania *et al* [3] and Bakheit[4]) The contrary findings may be due to the different set of breeding materials In the present study many tall genotypes were found to give low fodder yield as compared to those genotypes having comparatively less plant height giving high fodder yield due to better tillering ability, corky stem and more number of leaves per plant

Elaborative study of results based on genotypic correlations in relation to phenotypic correlations, indicated that both at phenotypic as well as at genotypic levels, green fodder yield had significant and positive correlation with leaf length and dry matter yield However, dry matter yield showed positive and significant correlation at phenotypic and genotypic levels only with plant height

Green fodder yield was observed to show contrasting relation with days to 50% flowering at phenotypic and genotypic levels Similarly, for other inter-character correlations viz , number of leaves and internodal length, the correlation coefficient were observed in contrasting direction. This may be because of effect of environment which seems to cause either increasing or reducing effect in expressing the ultimate correlation at phenotypic level

For rest of other inter-character correlations, the phenotypic and genotypic correlation coefficients, in general, did not differ in direction however, the magnitude was in general higher for genotypic correlation coefficient as compared to phenotypic coefficients This may be due to the masking role of environment in the actual expression of characters thereby leading to inflated observed values of phenotypic correlation coefficients even with high intensity of correlation at genotypic levels (Swamp and Chaugale[5], Vasudeva Rao[6] and Panchal *et al.*[7])

The plant height was significantly and positively correlated with dry matter yield while leaf width had significant and negative correlation with dry matter yield as is earlier reported by Bakheit[4].

Positive association of days to 50% flowering with plant height indicated that delay in flowering indirectly affected the expression of this character through ultimate increase in height Plants which are forced to grow taller due to delayed flowering also

have increased expression of their number of leaves, number of nodes per plant, stem diameter, T.S.S. %, shootfly infestation and protein content, which is exhibited by the significant and high positive association of these characters with days to 50% flowering

Since in any programme of forage sorghum improvement, the dry matter yield is considered more stable trait than green fodder yield, therefore, with the objective of improving the dry matter yield, the selection programme may be focused on selection of tall plant with medium thick stem and high number of long leaves Highly significant positive correlation of stem diameter with T.S.S. % and positive correlation with protein % shall also be exploited through above selection programme in improving t he quality of fodder in terms of palatability and nutritional values, simultaneously, with increased dry matter yield.

Non significant correlation coefficient of shootfly infestation with all other characers except days to 50% flowering indicates that the characters taken in this study had little effect on imparting resistance/susceptibility to shootfly infestation. Significant positive correlation with days to 50% flowering is indicative of the fact that genotypes with late flowering (maturity) are more prone to shootfly attack whereas the early genotypes as result of escape mechanism might have shown the resistance/less infestation

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